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Studies in Lipolysis by Fungi

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Optimum conditions for the lipolytic activity of three strains of *Aspergillus* — *A. awamori* Nakaz., *A. flavus* Link ex Fr. and *A. fumigatus* Fresen — on groundnut oil have been determined; *A. awamori* Nakaz. has been found to be the most promising strain. The addition of sugars retards the lipolytic activity of all the three strains though they show more profuse growth. There are indications of synthesis of fat when glucose or sucrose is added to the medium. Nitrogen and phosphorus are found to influence their growth as well as lipolytic activity; nitrate nitrogen is preferred to other forms of nitrogen.

Studies on the lipolytic activity of the three strains of *Aspergillus* on eight fats, in oil-in-water emulsions, indicate that the extent of hydrolysis varies with the fat. When water-in-oil emulsions, which can hold much more fat, are used, the fats are hydrolysed to a less extent, but the absolute amount of oil hydrolysed per 100 g. of the medium is 3-6 times more in the case of water-in-oil emulsion than in the case of oil-in-water emulsion. When submerged culture technique is used, the extent of fat splitting is lowered considerably.

Changes brought about in analytical characteristics of fats during their lipolysis by the strains of *Aspergillus* indicate an increase in the saponfication value and a decrease in all other values except Polenske value which remains more or less unchanged. The action of fungi indicates a strong reducing action. Glycerol is consumed by all the three strains but to varying extents.

IN a previous communication[1], the isolation and characteristics of the three strains of *Aspergillus* — *A. awamori* Nakaz., *A. flavus* Link ex Fr. and *A. fumigatus* Fresen — have been described. During a period of five days these fungi were shown to hydrolyse 60, 41 and 28 per cent of groundnut oil while at the same time consuming about 15, 12 and 21 per cent of the oil. Various factors like substrate concentration[2,3], temperature,[2,4,5], *p*H[4-6], inorganic[5,6] and organic nutrients[6,7], etc., are known to influence the metabolism and enzymic activities of fungi. Some of these factors in respect of the three strains of *Aspergillus* are evaluated in this paper.

Experimental procedure

Optimum conditions for hydrolysis of groundnut oil

Czapek's medium[8] containing groundnut oil instead of sugar was used. Addition of 2 per cent agar helped in emulsifying the oil as an oil-in-water emulsion and setting the medium. The *p*H of the medium so prepared was about 5·0. Different nutrients, acid or alkali, were added to the medium as required. The groundnut oil used had acid value, 2·00; sap. value, 190·0; R.M. value, 0·51; Polenske value, 0·33; iodine value (Wijs), 97·8; and acetyl value, 15·1.

Twenty ml. portions of the sterilized medium were inoculated with 2 ml. of spore suspensions prepared from freshly grown cultures of the three strains of *Aspergillus*. The inoculated medium was poured into sterile petri dishes and allowed to set. Preliminary studies had shown that during 5 days of growth the fungi sporulated well and possessed maximum lipolytic activity. On the sixth day, the fungal growth with the medium was lifted off each petri dish, dried and extracted with ethyl ether in a soxhlet. The loss

in weight in the ether extract, over that of the ether extract from an uninoculated petri dish, gave the oil consumed by the fungus. Aliquots of the ether extract were dissolved in neutral alcohol-benzene (1 : 1) mixture and free acidity estimated (as per cent oleic acid) by titrating with $N/10$ alkali. The increase in acidity over the blank (uninoculated) was taken as a measure of the extent of hydrolysis or the lipolytic activity. The extent of hydrolysis per unit consumption of oil or the metabolic ratio was calculated by dividing the percentage of fat hydrolysed by the percentage of oil consumed, all expressed on original oil content.

Hydrolysis of different fats

Studies on the hydrolysis by these three fungi under their optimal conditions, of seven other fats — coconut, castor, sesame, safflower and olive oils and of ghee (butter fat; buffalo) and hydrogenated vegetable oil (' Dalda ') — using oil-in-water and water-in-oil emulsions, and surface and submerged cultures were carried out. Groundnut oil was also included for comparison.

Oil-in-water emulsion was prepared as described earlier. Water-in-oil emulsion was prepared as follows:

(a) *Liquid emulsion with groundnut and castor oils* — Two per cent monoglyceryl stearate was dissolved in the oil to which the requisite amount of water containing nutrients and spore suspension was added and the mixture mixed well in a Waring blender for 10 min.; the mixture was further homogenized in a hand homogenizer. The emulsion contained about 50 per cent fat.

(b) *Solid butter-type emulsion with vanaspati and coconut oil* — About 1·5 per cent of monoglyceryl stearate was dissolved in the molten fats and churned with excess water containing the nutrients and spore suspension. Within 15 min. of churning, the ' butter ' separates out. These solid emulsions were found to hold more than 80 per cent of fat.

Submerged cultures — The modified Czapek's medium described earlier was used, with only 1 per cent agar instead of 2 per cent. This helps emulsification but is not enough to solidify the medium. Twenty ml. portions of the modified medium were placed in a conical flask and inoculated with the desired spore suspension. The flasks were aerated by the use of a rotary shaker at 60 r.p.m. Only groundnut oil was used for these studies.

A control without spores was run with each set. As before, at the end of 5 days, the media were dried and extracted with ether, and the metabolic ratios determined.

Effect of fungal lipolysis on oil characteristics

As fungi will, as a result of hydrolysis, modify the properties of the oil, the changes in the oil characteristics were examined using groundnut, coconut, castor and safflower oils and ghee.

Acid, iodine (Wijs), saponification, R.M., Polenske and acetyl values were determined by standard methods[9]. Peroxide value was estimated by the method of Dastur and Lea[10] and expressed as ml. of $N/500$ sodium sulphite per gram of fatty material. Naegamwala's[11] method was employed for the determination of aldehyde value. The aldehyde was reacted with excess of sodium bisulphite in a neutral medium, the excess of sulphite oxidized by iodine solution in an acid medium at a pH below 2, and titrated with standard iodine in an alkaline medium (pH 9·5) of the sodium sulphite formed by the dissociation of the aldehyde sulphite compound. The results are expressed as ml. of $N/500$ iodine per gram of fat.

The estimation of glycerol was carried out by the method of Hoepe and Treadwell[12] and as modified by Chinoy[13]. About 0·4-0·5 g. of glycerol sample was diluted to 50 ml. with water to which were added 2·5 to 3 g. potassium periodate. The mixture was shaken for 45 min. in a glass-stoppered bottle at room temperature (25° to 30°C.). The excess KIO_4 was filtered off and the volume of filtrate made up to 100 ml. The liberated formic acid was titrated in 20 ml. aliquots of reaction mixture against $N/10$ NaOH using methyl red as indicator.

$$C_3H_8O_3+2KIO_4=2HCHO+HCOOH+H_2O+2KIO_3$$

As the oxidation was not complete, a factor (1·0/0·9) was employed throughout for the calculation of the amount of glycerol.

Results and discussion

Optimum conditions for hydrolysis of groundnut oil

The results of different experiments are recorded in Tables 1 and 2.

Oil concentration — More luxuriant growth of the fungi was observed with increasing concentration of oil. The metabolic ratio of *A. fumigatus* remained more or less unchanged at different concentrations of the oil except at the lowest concentration caused probably by shortage of nutrients. The higher metabolic ratios of the other two fungi at higher oil concentrations point to greater hydrolysis per unit consumption of oil.

Temperature — It was observed that *A. fumigatus* belongs to the thermophilic group while *A. flavus* and *A. awamori* are mesophills.

pH — The acid range is more suitable for lipolytic activity. The metabolic ratio of *A. awamori* is

TABLE 1 — EFFECT OF VARIOUS FACTORS ON LIPOLYTIC ACTIVITY OF DIFFERENT STRAINS OF *ASPERGILLUS*

	Lipolytic activity (oleic acid), %			Oil consumption %			Metabolic ratio		
	A. awamori	*A. flavus*	*A. fumigatus*	*A. awamori*	*A. flavus*	*A. fumigatus*	*A. awamori*	*A. flavus*	*A. fumigatus*
Oil conc., %									
4·2	43·10	30·55	18·98	20·16	15·05	25·66	2·14	2·03	0·74
7·2	51·10	35·50	28·03	17·26	13·76	20·61	2·96	2·58	1·36
9·2	60·04	41·04	24·47	15·44	12·18	18·13	3·89	3·37	1·35
15·0	51·00	31·06	19·23	13·79	7·67	13·54	3·70	4·05	1·42
Temp., °C.									
10	0·93	0·90	0·58	0·42	0·53	0·38	2·22	1·69	1·53
27	49·30	38·30	21·53	8·56	10·88	12·23	5·76	3·52	1·76
34	51·56	43·70	25·53	9·84	12·78	15·47	5·24	3·42	1·65
38	60·23	41·13	28·54	15·29	12·17	20·83	3·94	3·38	1·37
42	44·90	20·82	31·67	9·20	7·23	22·15	4·88	2·88	1·43
46	14·98	4·28	25·76	3·30	3·01	15·71	4·54	1·42	1·64
*p*H									
4·0	50·98	36·31	23·00	10·18	9·84	15·03	5·01	3·69	1·53
5·0	59·50	40·94	27·85	15·18	12·15	20·48	3·92	3·37	1·36
6·0	61·13	36·90	32·97	16·34	10·51	23·55	3·74	3·51	1·40
7·0	62·42	34·56	26·13	17·10	10·02	16·23	3·65	3·45	1·61
7·5	56·84	31·04	22·52	13·50	7·96	14·35	4·21	3·90	1·57
8·5	49·89	27·58	19·42	12·11	7·00	12·21	4·12	3·94	1·59
Nitrogen ($NaNO_3$), p.p.m.									
165	57·68	36·44	17·17	9·23	9·01	11·06	6·25	4·05	1·56
330	62·67	44·01	31·50	15·53	12·19	20·63	4·04	3·61	1·53
495	—	—	33·56	—	—	21·17	—	—	1·59
660	64·98	44·07	—	17·24	12·25	—	3·77	3·60	—
Phosphorus (KH_2PO_4), p.p.m.									
Nil	28·26	28·10	14·81	4·37	5·83	7·09	6·47	4·82	2·09
114	49·22	39·76	22·01	8·05	9·48	12·95	6·12	4·20	1·70
225	63·18	44·12	31·57	15·64	12·14	20·64	4·04	3·63	1·53
342	63·65	44·74	33·00	15·90	12·26	22·07	4·00	3·65	1·50

TABLE 2 — COMPARISON OF OPTIMUM CONDITIONS FOR HYDROLYSIS OF OIL BY DIFFERENT STRAINS OF *ASPERGILLUS*

	A. awamori	*A. flavus*	*A. fumigatus*
Conc. of oil, %	9·2	9·2	7·2
Temp., °C.	38·0	34·0	42·0
*p*H	7·0	5·0	6·0
Conc. of nitrogen, p.p.m.	660·0	330·0	495·0
Conc. of phosphorus, p.p.m.	228·0	228·0	342·0
Potassium and magnesium	No influence on growth or lipolytic activity		
Addition of sugars	Lipolytic activity of all strains considerably reduced; oil not consumed but a small quantity of fat was synthesized		

minimum at the optimum *p*H (6·0-7·0) while for the other two fungi the ratios are constant over the entire range of *p*H tried.

Minerals — Nitrogen and phosphorus influenced the lipolytic activity. Of the various forms of nitrogen tried all the three fungi preferred nitrate nitrogen. Addition of potassium or magnesium salts had no effect either on growth or lipolytic activity of all the three fungi (data not given in Table 1).

Carbon source — When glucose or sucrose was added to the medium, in addition to oil as carbon source, the lipolytic activity of the organisms was very much reduced and oil consumption was nil (data not shown in Table 1). On the other hand, there were indications of oil synthesis, at the expense of sugars.

The optimum conditions for maximum hydrolysis of the three strains of fungi are given in Table 2.

Hydrolysis of different fats

The results of different experiments are given in Tables 3-6.

Oil-in-water emulsion — The extent of hydrolysis by the three different strains varies with different oils. *A. awamori* brings about maximum hydrolysis of groundnut oil, *A. flavus* of coconut oil and *A. fumigatus* of ghee and coconut oil (Table 3). The metabolic ratios of *A. flavus* and *A. fumigatus* are all lower

TABLE 3 — HYDROLYSIS AND CONSUMPTION OF DIFFERENT FATS BY DIFFERENT STRAINS OF *ASPERGILLUS* IN OIL-IN-WATER EMULSIONS

Fat	Lipolytic activity (oleic acid), %			Oil consumption %			Metabolic ratio		
	A. awamori	*A. flavus*	*A. fumigatus*	*A. awamori*	*A. flavus*	*A. fumigatus*	*A. awamori*	*A. flavus*	*A. fumigatus*
Groundnut oil	60·44	41·40	38·77	15·30	12·40	28·30	3·95	3·34	1·37
Coconut oil	32·45	48·10	40·82	5·10	14·80	29·80	6·36	3·25	1·37
Castor oil	49·82	37·39	37·80	9·01	11·13	27·19	5·53	3·36	1·39
Butter fat (buffalo)	42·42	44·87	40·20	8·52	13·56	28·50	4·98	3·31	1·41
Hydrogenated fat (*Dalda*)	41·90	35·70	36·16	8·15	10·85	26·20	5·14	3·29	1·38
Sesame oil	26·38	30·30	26·20	4·72	9·65	19·41	5·59	3·14	1·35
Safflower oil	37·25	37·47	32·17	5·94	11·46	23·49	6·27	3·27	1·37
Olive oil	45·90	39·04	35·00	8·91	11·83	25·37	5·15	3·30	1·38

TABLE 4 — HYDROLYSIS OF FATS BY DIFFERENT STRAINS OF *ASPERGILLUS* IN WATER-IN-OIL EMULSIONS

Fat	Lipolytic activity (oleic acid), %			Oil consumption %			Metabolic ratio		
	A. awamori	*A. flavus*	*A. fumigatus*	*A. awamori*	*A. flavus*	*A. fumigatus*	*A. awamori*	*A. flavus*	*A. fumigatus*
Liquid emulsion (50% fat in medium)									
Groundnut oil	37·70	24·28	26·29	5·80	3·12	6·46	6·50	7·78	4·07
Castor oil	32·82	21·10	24·82	4·75	2·96	6·10	6·91	7·13	4·07
Solid emulsion (80-84% fat in medium)									
Hydrogenated fat	25·34	14·86	17·02	3·41	1·92	3·93	7·43	7·74	4·33
Coconut oil	17·53	15·39	13·44	2·13	2·49	4·80	8·23	6·18	2·80

TABLE 5 — FREE FATTY ACIDS PRODUCED BY DIFFERENT STRAINS OF *ASPERGILLUS* IN THE TWO TYPES OF EMULSIONS

Fat	Fatty acids *g./100 g. medium*			Oil consumption *g./100 g. medium*			Metabolic ratio		
	A. awamori	*A. flavus*	*A. fumigatus*	*A. awamori*	*A. flavus*	*A. fumigatus*	*A. awamori*	*A. flavus*	*A. fumigatus*
Groundnut oil									
Oil/water	5·60	3·84	2·85	1·41	1·20	2·03	3·95	3·34	1·37
Water/oil	18·80	12·11	13·14	2·89	1·55	3·22	6·50	7·78	4·07
Castor oil									
Oil/water	4·66	3·48	2·78	0·82	1·02	1·96	5·53	3·36	1·39
Water/oil	16·47	10·60	12·50	2·38	1·48	3·06	6·91	7·13	4·07
Hydrogenated fat (*Dalda*)									
Oil/water	3·92	3·31	2·67	0·75	0·99	1·89	5·14	3·29	1·38
Water/oil	20·33	11·91	13·68	2·73	1·54	3·15	7·43	7·74	4·33
Coconut oil									
Oil/water	3·06	4·66	3·01	0·46	1·36	2·15	6·36	3·25	1·37
Water/oil	14·78	12·97	11·38	1·79	2·10	4·04	8·22	6·18	2·80

and more or less the same, whichever fat is used, indicating a proportionality between lipolytic activity and growth irrespective of the nature of fat. On the other hand, the metabolic ratio of *A. awamori* is both higher for any particular fat than the other strains and more variable from fat to fat. This strain seems to favour lipolytic activity to growth.

Water-in-oil emulsion — In oil-in-water emulsion a maximum of only 15-20 per cent oil could be incorporated. With more fat, the emulsion becomes unstable and soggy. The water-in-oil emulsion can hold much larger quantities of oil, from about 50 per cent in the liquid emulsions to more than 80 per cent in the solid butter-type emulsions. With larger quantities of fat in the medium, better hydrolysis of the fat by the fungi might be expected.

The data given in Table 4 show that with a higher fat content in the water-in-oil emulsion, the percentage hydrolysis of all the fats investigated by all the strains of *Aspergillus* is very much less than when

TABLE 6 — EXTENT OF HYDROLYSIS OF GROUNDNUT OIL UNDER SUBMERGED CULTURE CONDITIONS BY DIFFERENT STRAINS OF *ASPERGILLUS*

	A. awamori		*A. flavus*		*A. fumigatus*	
	Submerged	Surface	Submerged	Surface	Submerged	Surface
Lipolytic activity (oleic acid), %						
5 days	27·84	60·66	15·00	43·33	10·46	29·76
10 days	34·27	—	19·92	—	14·75	—
Oil consumption, %						
5 days	2·06	12·20	1·23	10·50	1·01	16·42
Mat weight, mg.						
5 days	142·00	472·00	90·00	345·00	105·00	406·00
Lipolytic activity (oleic acid), %						
With 0·5% calcium chloride	36·24	—	21·85	—	10·69	—
With 0·1% ferrous sulphate	35·30	—	22·25		10·68	—
Mat weight, mg.						
With 0·5% calcium chloride	156·00	—	101·00	—	100·00	—
With 0·1% ferrous sulphate	160·00	—	106·00	—	111·00	—

the fats were in the form of oil-in-water emulsions. The amount of oil consumption is also reduced, thereby increasing the metabolic ratios. However, the calculated absolute quantities of oil hydrolysed per 100 g. medium (Table 5) are 3 to 6 times greater with the water-in-oil emulsions than with the oil-in-water emulsions by all the three strains of fungi. The increase may be due either to more luxuriant growth or to more lipase secretion or to both, consequent on the availability of more nutrient materials to the fungi.

Submerged culture — This method gives homogeneous physiological conditions by elimination of differential diffusion effects. The amount of fat in the medium is just the same (15-20 per cent) as in the oil-in-water emulsions studied earlier.

It will be seen from the results given in Table 6 that the extent of hydrolysis is very low with all the three strains. Allowing the growth to proceed for 10 days does not seem to help. Growth under the submerged conditions is also very poor, the mat weights being a fourth of the mat weights of the fungus when grown on the surface of solid media. In the earlier studies it was noticed that lipolytic activity increased when reaching sporulation. Under submerged conditions, there was no sporulation even after 10 days. Gilbert and Hickey[14] report that a high calcium content favoured conidia formation in two strains of *Penicillium*. Foster *et al.*[15] observed with the same strains that 0·05-0·1 per cent of Fe^{2+} encouraged sporulation while 0·2 per cent Fe^{2+} inhibited growth. The addition of either calcium chloride or ferrous sulphate to the submerged culture media with the present strains yielded no response either in growth or in hydrolytic activity, though there was slight sporulation.

TABLE 7 — ANALYTICAL CHARACTERISTICS OF GROUNDNUT OIL BEFORE AND AFTER LIPOLYSIS BY DIFFERENT STRAINS OF *ASPERGILLUS*

	Original oil	After lipolysis by		
		A. awamori	*A. flavus*	*A. fumigatus*
Acid val.	6·36	167·30	110·40	130·30
Sap. val.	190·00	220·00	199·80	208·50
R.M. val.	0·51	0·06	0·31	0·21
Polenske val.	0·33	0·87	0·30	0·32
Iodine (Wijs) val.	97·79	85·82	92·50	89·50
Peroxide val.	7·65	4·45	6·55	6·00
Aldehyde val.	1·55	0·50	1·00	0·55
Acetyl val.	15·61	10·05	12·14	11·09
Mean mol. wt of fatty acids	281·00	242·50	269·30	256·80

Effect of fungal lipolysis on oil characteristics

Experiments were conducted using the three strains of *Aspergillus* on groundnut, coconut, castor and safflower oils and butter fat or ghee. In addition to the expected large increase in acid value due to hydrolysis, there were changes observed in the other analytical characteristics. As the trend of these changes were more or less the same with all the oils tried, only the data for groundnut oil and castor oil are reported in Tables 7 and 8.

Analytical characteristics — After lipolysis by any of the three strains, all the oils tried showed an increase in saponification value and a decrease in all other values except Polenske value which remained more or less unchanged. The decrease in iodine value may be due either to fission of the double bond or their saturation. Increase in saponification value also indicates formation of low molecular weight fatty acids. Fission of a double bond does not appear to be

TABLE 8 — ANALYTICAL CHARACTERISTICS OF CASTOR OIL BEFORE AND AFTER LIPOLYSIS BY DIFFERENT STRAINS OF *ASPERGILLUS*

	Original oil	After lipolysis by		
		A. awamori	*A. flavus*	*A. fumigatus*
Acid val.	5·10	115·60	90·60	111·50
Sap. val.	182·00	197·60	184·10	195·20
R.M. val.	1·90	1·35	1·09	1·45
Polenske val.	—	—	—	—
Iodine (Wijs) val.	86·00	82·00	85·10	81·80
Peroxide val.	6·35	4·05	4·50	4·25
Aldehyde val.	0·95	0·35	0·60	0·50
Acetyl val.	149·60	117·60	122·50	114·80
Mean mol. wt of fatty acids	293·00	260·00	275·90	265·10

TABLE 9 — FATE OF GLYCEROL DURING FUNGAL LIPOLYSIS OF GROUNDNUT OIL

(*Values expressed in g. per* 100 *g. of groundnut oil*)

Incubation period *days*	Combined glycerol	Free glycerol in hydrolysed product	Combined glycerol (calc. from sap. val.)	Glycerol released per day	Glycerol consumed per day
		A. awamori			
0	9·985	Traces	9·972	nil	nil
1	9·813	do	9·762	0·172	0·172
2	7·426	1·248	6·366	2·387	1·139
3	4·608	2·645	4·090	2·818	1·421
4	4·010	1·601	3·618	0·598	1·642
5	3·520	0·601	3·456	0·490	1·490
		A. flavus			
0	9·985	Traces	9·972	nil	nil
1	9·680	0·160	9·546	0·305	0·145
2	9·104	0·141	0·019	0·576	0·595
3	7·160	1·067	6·812	1·944	1·018
4	6·040	0·893	5·912	1·120	1·294
5	5·610	0·067	5·584	0·430	1·256
		A. fumigatus			
0	9·985	Traces	9·972	nil	nil
1	9·900	do	9·782	0·085	0·085
2	9·490	do	9·212	0·410	0·410
3	8·912	do	8·764	0·578	0·578
4	7·814	0·181	7·666	1·098	0·917
5	7·012	Traces	6·951	0·802	0·802

feasible for two reasons. Fission involves formation of new carboxylic groups which is not warranted under the reducing conditions existing during fungal growth and lipolysis as indicated by the destruction of peroxides and aldehydes. Secondly, the increase in the saponification value is not sufficient to account for the entire decrease in iodine value, assuming that the fission of each double bond corresponds to the formation of two carboxylic groups. Hence, the reduction in iodine value during fungal lipolysis may be presumed to be due to saturation of double bonds which is more likely under the reducing conditions. Such reducing conditions suggest the probable utilization of part of the auxiliary oxygen of the fat molecule itself for the growth of the fungus. This offers a probable explanation for the preference of the nitrate form of nitrogen by the fungi.

The increase in saponification value is not accompanied by a concomitant increase in either the R.M. value or the Polenske value. Evidently, the lower fatty acids formed are of higher molecular weight than the steam volatile acids.

Glycerol — The amount of glycerol released per day was calculated from the day-to-day decrease in combined glycerol. The difference between the glycerol released and the free glycerol actually present indicates the amount of glycerol consumed by the fungus (Table 9).

A. fumigatus seems to consume all the glycerol as fast as it is released. *A. awamori* consumed the minimum amount of glycerol as can be seen from the amounts of free glycerol left in the medium. On the third day 2·645 g. of glycerol is present in the medium used for *A. awamori* as compared to only 1·067 g. of glycerol in the medium used for *A. flavus*. The calculated quantities (from saponification value) of combined glycerol on all days are less than the estimated quantities of combined glycerol. This indicates that the triglyceride is not hydrolysed directly to free glycerol and fatty acids but stepwise through di- and monoglyceride stages.

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Studies on the Metabolism of Butyrate in Scurvy

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The effect of feeding sodium butyrate on the tissue contents of citric acid, lactic acid and cholesterol, total body contents of cholesterol and lipid, liver glycogen and blood ketone bodies has been studied in normal, scorbutic and insulin treated scorbutic guinea-pigs. Citric and lactic acid contents of tissues have been found to increase greatly in scorbutic animals. No change in blood ketone bodies has been observed. Liver glycogen and total body lipid are significantly reduced and total body cholesterol is enormously increased in the scorbutic animals. The overall effect of insulin treatment is to restore to normal the operation of tricarboxylic acid cycle, to promote glycogenesis and lipid synthesis, and to reduce total body cholesterol. The results provide additional evidences to support the contention that insulin insufficiency might be responsible for the disturbed carbohydrate and fat metabolism observed in scorbutic guinea-pigs.

METABOLISM of acetate and/or acetoacetate has been studied to some extent in scorbutic guinea-pigs. Becker *et al.*[1] reported that liver and adrenals of scorbutic guinea-pigs fed labelled acetate contain cholesterol with higher specific activity. Banerjee and Singh[2] showed that total body cholesterol is greatly increased in scorbutic guinea-pigs and prolonged treatment of scorbutic animals with small doses of insulin lowered the cholesterol content to normal level. Debsons *et al.*[3] found that liver tissue from ascorbic acid-deficient guinea-pigs produces less ketone bodies than normal controls. The entrance of acetate in the tricarboxylic acid cycle does not seem to be adversely affected, in as much as feeding of butyrate to scorbutic guinea-pigs greatly increases the citric acid excretion in urine[4,5]. But the further operation of this metabolic pathway has been shown to be defective as citric, lactic and malic acids accumulate in the tissues of scorbutic guinea-pigs[6]. In view of these observations it was considered of interest to study the metabolism of an increased pool of acetate and/or acetoacetate, brought about by feeding sodium butyrate, in scorbutic guinea-pigs.

Materials and methods

Male guinea-pigs, weighing 250-300 g., were fed green grass, soaked gram and scorbutogenic diet[7] for 6-7 days. Those animals that grew well were selected and divided into several groups, each consisting of one normal, one scorbutic and one insulin treated scorbutic animal. The animals were fed the basal scorbutogenic diet[7] and two drops of a concentrate of vitamins A and D twice a week. The normal animal of each group was fed 5 mg. of ascorbic acid daily. Insulin (Lilly) was injected subcutaneously to the animals intended for insulin treatment with a dose increasing from 0·1 to 0·3 unit per 100 g. body weight per day from the beginning of the second week on scorbutogenic diet. The normal and the insulin treated scorbutic guinea-pigs in each group were allowed the amount of food consumed by the corresponding scorbutic guinea-pig. All the animals were fed daily, in three divided doses, a neutral solution of butyric acid (1 ml. per 100 g. body weight) from the tenth day on scorbutogenic diet till the 24th day when signs of scurvy were apparent.

Estimation of tissue citric and lactic acids content, liver glycogen and blood ketone bodies — On the 25th day, after an overnight fast, the animals were stunned and decapitated. Blood was collected from the neck veins in an oxalated tube. Tissues were quickly removed, wrapped in filter paper to remove the adherent blood and dropped in weighed beakers containing 1*M* trichloroacetic acid. The beakers were weighed again to obtain the weight of the tissues taken. Citric acid was estimated by the method of Speck *et al.*[8] and lactic acid by the method of Barker and Summerson[9]. The tissues taken were liver, kidney, brain and skeletal muscle. A part of the liver was dropped in a weighed beaker containing

30 per cent potassium hydroxide. Glycogen was precipitated by the method of Grattan and Jensen[10] and the reducing sugar in the hydrolysed precipitate was estimated by the method of Hagedorn and Jensen[11]. Blood was deproteinized with barium hydroxide and zinc sulphate and total ketone bodies were estimated by the method of Behre[12].

Estimation of total body cholesterol and total body lipid — The animals were fasted overnight and killed on the 25th day by stunning and decapitation. Fur was plucked off, gastro-intestinal tract was washed, the whole body minced, weighed and dried at 80°C. for 20 hr. The dried body was powdered and extracted over a period of 24 hr with petroleum ether in a soxhlet apparatus. The extract was made to 100 ml. and a portion was dried to constant weight to give total body lipid content. Total cholesterol was estimated by the method of Sobel and Mayer[13] in another portion of the extract after proper dilution.

Estimation of cholesterol content of different tissues — After an overnight fast the animals were stunned and decapitated. Blood was collected from the neck veins. Tissues were removed, blotted with filter paper and thinly spread with the blunt end of the scalpel on previously weighed filter papers. These were weighed again and dried at 60°C. for 10 hr. The dried sample was extracted with acetone in a continuous extractor for 10 hr and total cholesterol was estimated in the extract by the method of Sobel and Mayer[13].

Results

An increase in citric acid content of tissues of scorbutic guinea-pigs was observed (Table 1). Insulin treatment brought the level to normal. Similarly, lactic acid content of the tissues was higher in scorbutic animals and insulin treatment had a similar effect (Table 2). The level of total ketone bodies in blood was higher in scorbutic guinea-pigs than normal and insulin treated scorbutic guinea-pigs but the difference was not found to be statistically significant (Table 3). Liver glycogen was significantly reduced in scorbutic animals. Insulin treatment effected only a partial restoration to normal level (Table 4). The total cholesterol content of different tissues (Table 5) showed that there was no change in liver and kidney but brain cholesterol was significantly decreased, and intestine, skin and blood

TABLE 1 — CITRIC ACID CONTENT OF TISSUES OF EXPERIMENTAL ANIMALS FED ON BUTYRATE

[*Eight animals used in each group and fed on* 15 *per cent butyrate* (1 *ml.*/100 *g. body wt*) *for* 15 *days; values expressed in mg.*/100 *g. wet wt of tissue*]

Animals	Liver	Kidney	Brain	Muscle
Normal	4·48±0·55	16·94±1·63	7·63±0·64	5·18±0·61
Scorbutic	11·69±0·79	27·76±1·19	15·35±1·05	15·27±1·79
Insulin treated scorbutic	4·46±0·70	16·35±1·05	9·32±0·92	8·14±0·87
	STATISTICAL ANALYSIS (*t* VALUES)			
Between normal and scorbutic	7·46*	5·37*	6·26*	5·31*
Between normal and insulin-treated scorbutic	0·02	0·31	1·51	2·83*
Between scorbutic and insulin treated scorbutic	6·81*	7·21*	4·28*	3·58*

*Significant at 5 per cent level.

TABLE 2 — LACTIC ACID CONTENT OF TISSUES OF EXPERIMENTAL ANIMALS FED ON BUTYRATE

[*Eight animals used in each group and fed on* 15 *per cent butyrate* (1 *ml.*/100 *g. body wt*) *for* 15 *days; values expressed in mg.*/100 *g. wet wt of tissue*]

Animals	Liver	Kidney	Brain	Muscle
Normal	92·30±10·12	179·10±16·20	178·30± 9·95	176·70±17·17
Scorbutic	214·20±11·55	315·50±15·91	292·60±16·00	284·70±12·24
Insulin treated scorbutic	86·40± 5·23	155·20±12·89	144·30±12·19	143·10±16·21
	STATISTICAL ANALYSIS (*t* VALUES)			
Between normal and scorbutic	7·93*	6·00*	6·06*	5·12*
Between normal and insulin treated scorbutic	0·52	1·15	2·16*	1·42
Between scorbutic and insulin treated scorbutic	10·00*	7·82*	7·37*	6·97*

*Significant at 5 per cent level.

TABLE 3 — TOTAL BODY CHOLESTEROL, TOTAL BODY LIPID AND BLOOD KETONE BODIES CONTENT OF EXPERIMENTAL ANIMALS FED ON BUTYRATE

[*Eight animals used in each group and fed on* 15 *per cent butyrate* (1 *ml.*/100 *g. body wt*) *for* 15 *days; values given are mean* ± *S.E.*]

Animals	Total body cholesterol (*mg.*/100 *g. wet wt*)	Total body lipid (*g.*/100 *g. wet wt*)	Blood ketone bodies (*mg. acetone*/100 *ml.*)
Normal	121·00± 9·98	6·69±0·75	1·115±0·093
Scorbutic	277·00±11·56	2·67±0·61	2·206±0·324
Insulin treated scorbutic	144·00±14·22	6·09±0·89	1·128±0·162
STATISTICAL ANALYSIS (*t* VALUES)			
Between normal and scorbutic	10·21*	4·16*	1·111
Between normal and insulin treated scorbutic	1·32	0·51	0·012
Between scorbutic and insulin treated scorbutic	7·26*	3·15*	1·770

*Significant at 5 per cent level.

TABLE 4 — GLYCOGEN CONTENT OF LIVER OF EXPERIMENTAL ANIMALS FED ON BUTYRATE

[*Eight animals used in each group and fed on* 15 *per cent butyrate* (1 *ml.*/100 *g. body wt*) *for* 15 *days; glycogen content expressed in terms of glucose equivalent in g.*/100 *g. wet wt of tissue*]

Normal	Scorbutic	Insulin treated scorbutic
2·816±0·627	0·641±0·048	1·181±0·239

STATISTICAL ANALYSIS (*t* VALUES)

Between normal and scorbutic	Between normal and insulin treated scorbutic	Between scorbutic and insulin treated scorbutic
3·46*	1·67	0·71

*Significant at 5 per cent level.

showed an enormous increase. Insulin treatment had no effect on brain cholesterol but intestine, skin and blood cholesterol content was brought to normal level. Total body cholesterol of scorbutic guinea-pigs showed a tremendous increase and insulin treatment lowered the cholesterol content to normal limit (Table 3). Total body lipid content was decreased in scorbutic guinea-pigs and insulin treatment had a beneficial effect (Table 3).

Discussion

The increased tissue content of citric acid in scorbutic guinea-pigs was attributed by Banerjee *et al.*[6] to its reduced oxidation through tricarboxylic acid cycle. Feeding of butyrate to scorbutic guinea-pigs has been shown to increase the citric acid excretion in urine to a great extent[4,5]. In the present investigation, however, citric acid levels in tissues of scorbutic animals are comparable to those reported earlier[6]. This may be expected because of the dynamic balance of different metabolic processes. Maximum tissue concentration must also play a certain part. Urinary excretions, however, would certainly represent the balance of the overall reactions. In conditions of butyrate feeding the system would be loaded with acetate and citrate formation would be one of the pathways. Further metabolism of this metabolite and the retention power of tissues would, to a great extent, influence the urinary excretion and tissue concentration of citrate. The observation of Banerjee and Singh[14] that scorbutic guinea-pigs excrete less malic acid in urine after the ingestion of citric acid suggests a depressed oxidation of citrate through tricarboxylic acid cycle. Though the possibility of an acceleration of reactions leading to citrate formation cannot be completely ruled out in the case of scorbutic guinea-pigs, it seems reasonable to assume that the depressed oxidation through tricarboxylic acid cycle is more

TABLE 5 — TOTAL CHOLESTEROL CONTENT OF TISSUES OF EXPERIMENTAL ANIMALS FED ON BUTYRATE

[*Eight animals used in each group and fed on* 15 *per cent butyrate* (1 *ml.*/100 *g. body wt*) *for* 15 *days; values for brain expressed in g.*/100 *g. wet wt, for blood in mg.*/100 *ml. and for the rest of tissues in mg.*/100 *g. wet wt*]

Animals	Liver	Kidney	Brain	Intestine	Skin	Blood
Normal	240·0±22·9	379·0±28·3	20·72±0·88	245·0±32·7	145·0±15·3	79·0±4·0
Scorbutic	279·0±24·2	354·0±32·1	16·75±0·51	403·0±36·4	240·0±20·6	109·0±4·2
Insulin treated scorbutic	256·0±19·2	334·0±36·1	17·80±0·41	255·0±27·8	154·0±15·4	77·0±4·5
STATISTICAL ANALYSIS (*t* VALUES)						
Between normal and scorbutic	1·11	0·58	3·80*	3·20*	3·70*	5·10*
Between normal and insulin treated scorbutic	0·52	1·02	2·99*	0·23	0·42	0·33
Between scorbutic and insulin treated scorbutic	0·74	0·46	1·61	3·20*	3·30*	5·20*

*Significant at 5 per cent level.

likely to be the cause of its high concentration in tissues.

It was suggested earlier[6] that high lactic acid content of tissues from scorbutic guinea-pigs may be due to reduced glycogen formation from this substrate. We have also observed reduced liver glycogen in scorbutic guinea-pigs. Capraro and Milla[15] found that injection of acetoacetate or β-hydroxybutyrate into dogs was followed by an almost immediate rise in blood lactate. Nath and Chakrabarti[16] reported that daily injections of acetoacetate for some time caused a gradual rise in blood lactate levels in rabbits. It is possible that this increased lactic acid content may be due to an increased pool of acetate and/or acetoacetate. In scurvy, acetate oxidation through tricarboxylic acid cycle is depressed. The data presented here show reduced lipid synthesis and glycogen formation. At least a part of this excess acetate may go to the formation of lactate. Enzyme systems have been described by Seaman[17] and Davies[18] in protozoa and pig heart which catalyse the oxidative condensation of two molecules of acetyl coenzyme A to produce succinate. Succinic acid would then give rise to pyruvic acid via oxaloacetic acid and ultimately pyruvic acid would form lactic acid. Normally the reaction is in favour of succinate splitting but it may just be possible that under conditions of increased available acetate aided by the scorbutic state, the reaction goes towards succinate synthesis. This would also explain the accumulation of malic acid in tissues from scorbutic guinea-pigs reported earlier[6]. This is in agreement with the observation of Banerjee and Biswas[19] that scorbutic animals excrete more pyruvic acid in urine and that succinate feeding increases the excretion to a great extent.

Insulin treatment of the scorbutic animals restored the tissue content of citric and lactic acids to normal level and improved liver glycogen. Thus, the reduced glycogenesis and depressed operation of tricarboxylic acid cycle seems to be due to lack of insulin. This is in agreement with the earlier observations of Banerjee *et al.*[4,6,19].

The total cholesterol content of different tissues of scorbutic guinea-pigs is affected to varying extents but the total body cholesterol is enormously increased. The different pathways open to the acetate pool of the body are: (i) metabolism through tricarboxylic acid cycle, (ii) ketone bodies formation, (iii) lipid synthesis and (iv) cholesterol synthesis. It has been shown that the operation of tricarboxylic acid cycle is depressed in scurvy. Lipid synthesis is also decreased. The data presented here show that blood ketone bodies levels are not significantly different in normal and scorbutic guinea-pigs. Metabolism of ketone bodies, therefore, does not seem to be much affected in scurvy. As such, the increased cholesterogenesis is likely to be due to the channelization of excess acetate towards cholesterol synthesis.

The whole effect of insulin treatment of the deficient animals seems to be to restore the normal operation of tricarboxylic acid cycle, enhance lipid synthesis, promote glycogenesis and thus open up all the pathways for their normal operation. Mukherjee and Sadhu[20] have observed the relationship between decreased oxidation of acetate and acetoacetate and cholesterol synthesis in alloxan diabetes. They suggested that continued depression of acetate and acetoacetate oxidation might result in channeling these metabolites towards cholesterol synthesis. In view of insulin insufficiency associated with scurvy[21], the possible role of insulin in the operation of tricarboxylic acid cycle[22] and the part played by insulin in decreasing the incorporation of acetate into cholesterol in alloxan diabetic rats[23], the results obtained in this study give further support to the contention that insulin insufficiency is more likely to be responsible for the observed disturbances in scorbutic guinea-pigs.

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Studies in Possible Oral Hypoglycaemic Agents: Part II*—Synthesis of Hydantoins & Hydantoic Esters by Alternate Methods

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The synthesis of 3-phenyl, 3-(2′,4′-dimethoxyphenyl), 3-octyl and 3-β-phenylethyl hydantoins as well as δ-undecyl hydantoic acid and ethyl-δ-(*p*-chlorophenyl) hydantoate, either by modification of Wada's method and/or by condensation of appropriate isocyanates with glycine, has been described.

THE observation that 3-phenyl, 3-(2′,4′-dimethoxyphenyl) and 3-octyl hydantoins and ethyl-δ-(*p*-chlorophenyl) hydantoate possess marked hypoglycaemic activity in rats[1] supported our suggestion[2] that the activity of some of the well-known oral hypoglycaemic agents, e.g. 5-isopropyl-2-*p*-aminobenzenesulphanilamido-1,3,4-thiadiazole (IPTD), N_1-*p*-aminophenylsulphonyl-N_2-*n*-butylurea (Carbutamide), N_1-toluene-*p*-sulphonyl-N_2-butylurea (Tolbutamide) and β-phenethylbiguanide (PEBG, DBI), was related to the presence, in the respective molecules of the thiourea, urea or guanidino moieties. It was considered desirable to prepare these compounds in sufficient quantities for detailed biological evaluation. Since phosgene, which was used previously for the synthesis of ethyl isocyanatoacetate, the key intermediate for the synthesis of the required hydantoates and the hydantoins, was no longer available in this country, it became necessary to devise alternate methods for the synthesis of the latter. Two routes, involving (a) preparation of the suitably substituted urea and its condensation with glycine by a modification of Wada's method[3] and (b) the preparation of the appropriate isocyanate by Curtius reaction followed by its condensation with glycine in presence of alkali[4], were tried and found useful. δ-Undecyl hydantoic acid and 3-β-phenylethyl hydantoin were also prepared by routes (a) and (b) respectively.

In order to obtain 2,4-dimethoxybenzoic acid, an intermediate in the synthesis of 3-(2′,4′-dimethoxyphenyl) hydantoin, the methylation of β-resorcylic acid according to the procedure of Venkataraman and Robinson[5] and Spatz and Koral[6] gave erratic results in our hands. A modification of an earlier method of Perkin and Schiess[7], involving the use of dimethyl sulphate, acetone and potassium carbonate, however, gave consistently good results. Further, whereas 3-(2′,4′-dimethoxyphenyl) hydantoin could be prepared in good yield by the method (a), the alternate procedure (b) did not give satisfactory results.

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Experimental procedure

All melting points are uncorrected.

2,4-*Dimethoxyphenylurea* — 2,4-Dimethoxyaniline hydrochloride was prepared from 2,4-dimethoxyaniline[8], m.p. 224° (decomp.). (Found: N, 7·71. $C_8H_{11}NO_2$, HCl requires N, 7·38%.) On treatment with potassium cyanate, according to the method of Hoffmann[9], it gave 2,4-dimethoxyphenylurea, m.p. 166-7°; yield 70 per cent. (Found: N, 14·78. $C_9H_{12}N_2O_3$ requires N, 14·28%.)

δ-(2′,4′-*Dimethoxyphenyl*) *hydantoic acid* — A mixture of glycine (0·46 g.), 2,4-dimethoxyphenylurea (1·67 g.) and barium hydroxide (1·34 g. in 50 ml. of water) was refluxed gently for 18 hr. The solution was filtered while hot and the filtrate acidified and kept overnight in the cold. The crystalline 2′,4′-dimethoxyphenyl hydantoic acid was collected and recrystallized from ethanol, m.p. 171-2°; yield 0·434 g. (46 per cent of the theoretical, calculated on the basis of glycine). (Found: N, 10·60. $C_{11}H_{14}N_2O_5$ requires N, 11·02%.)

3-(2′,4′-*Dimethoxyphenyl*) *hydantoin* — The above acid was cyclized to 3-(2′,4′-dimethoxyphenyl) hydantoin, according to the method reported earlier[2].

2,4-*Dimethoxybenzoic acid* — A solution of β-resorcylic acid (4·7 g.) in dry acetone (40 ml.) was refluxed, for 8 hr, in the presence of anhydrous potassium carbonate (20 g.) and during this period dimethyl

sulphate (4×5 ml.) was added in four lots. The reaction mixture was cooled and worked up in the usual way to obtain methyl-2,4-dimethoxybenzoate, b.p. 160-2°/13 mm.; yield 5 g. (83 per cent; Perkin and Schiess[7] obtained a yield of 30-40 per cent). The ester was hydrolysed with 10 per cent sodium hydroxide to 2,4-dimethoxybenzoic acid, m.p. 108°.

Octyl isocyanate — The method of Allen and Bell[10] for the preparation of isocyanates was followed. Pelargonyl chloride[11] (30 g.) in acetone (60 ml.) was reacted with a well-stirred solution of sodium azide (15·8 g.) in water (52 ml.) at 10-15°. The mixture was kept at this temperature for 1 hr. The organic layer was now separated, dried (anhydrous magnesium sulphate) and added slowly to dry benzene (150 ml.), previously warmed to 60°. After keeping the mixture at this temperature for 1 hr, it was worked up and octyl isocyanate obtained as a colourless liquid, b.p. 84°/4 mm.; yield 16 g.; 61 per cent.

The isocyanate is unstable at room temperature, and deposits a solid on keeping.

δ-(*n-Octyl*) *hydantoic acid* — Glycine (5 g.) was dissolved in a solution of potassium hydroxide (4 g.) in water (150 ml.) and treated with octyl isocyanate (10·32 g.) at room temperature. The resultant colourless precipitate (1·08 g.) was collected and crystallized from ethanol, m.p. 92°. This compound is presumably identical with dioctylurea. (Found: N, 9·65. $C_{17}H_{36}N_2O$ requires N, 9·85%.)

The filtrate, obtained above, was acidified with concentrated hydrochloric acid to obtain δ-(*n*-octyl) hydantoic acid; yield 11·55 g. (75 per cent). It crystallized from ethanol in shining flakes, m.p. 148-9°. (Found: N, 12·47. $C_{11}H_{22}N_2O_3$ requires N, 12·17%.)

3-*n*-Octyl hydantoin was prepared by cyclization of the acid by the usual method. It separated from ethanol-light petroleum (60-80°) in colourless plates, m.p. 94°. Mixed melting point with the authentic sample showed no depression.

δ-(*p-Chlorophenyl*) *hydantoic acid* — A mixture of *p*-chlorophenylurea (25·26 g.), glycine (8·42 g.) and barium hydroxide octahydrate (8·42 g. in 1000 ml. of water) was refluxed for 18 hr and subsequently acidified with hydrochloric acid and δ-(*p*-chlorophenyl) hydantoic acid obtained as colourless needles (ethanol), m.p. 191°; yield 31 per cent. (Found: N, 11·94. $C_9H_9N_2O_3Cl$ requires N, 12·25%.)

Ethyl-δ-(p-chlorophenyl) hydantoate — A mixture of *p*-chlorophenyl hydantoic acid (4·0 g.), absolute ethanol (120 ml.) and concentrated sulphuric acid (4 ml.; d. 1·84) was refluxed for 3 hr. Most of the solvent was distilled off at reduced pressure and the residue diluted with water and neutralized with finely powdered sodium bicarbonate. The resultant precipitate was filtered, washed with water and crystallized from ethanol to yield the ethyl ester as needles, m.p. 161-2°; yield 2·26 g. (51 per cent).

In another experiment the esterification of δ-(*p*-chlorophenyl) hydantoic acid gave some of the corresponding hydantoin in addition to the hydantoic ester.

δ-*Phenyl hydantoic acid* — (a) Phenyl isocyanate, prepared by the Curtius rearrangement of benzazide[12], was condensed with glycine according to the procedure of Biltz and Slotta[13] to give δ-phenyl hydantoic acid, m.p. 198°. (b) Aqueous barium hydroxide (0·8 g. in 80 ml. of water) was added to a mixture of phenylurea (3·65 g.) and glycine (1 g.) and the resulting suspension refluxed gently for about 16 hr. On working up the product in the usual way, δ-phenyl hydantoic acid (yield 0·8 g.; 31 per cent) was obtained.

Ring closure of the above acid furnished the corresponding 3-phenyl hydantoin in 87 per cent yield.

δ-*Undecyl hydantoic acid* — Undecyl isocyanate[10] was allowed to react with glycine according to the procedure of Delacoux *et al.*[14].

The δ-undecyl hydantoic acid so formed was isolated and crystallized from ethanol as a microcrystalline powder, m.p. 142° (effervescence). (Found: N, 9·79. $C_{14}H_{28}N_2O_3$ requires N, 10·29%.)

3-(β-*Phenylethyl*) *hydantoin* — This compound was prepared in an analogous way by the condensation of glycine with β-phenylethyl isocyanate[15] in the presence of dilute alkali. It crystallized from benzene in microcrystalline powder, m.p. 147-8°. (Found: N, 13·27. $C_{11}H_{12}N_2O_2$ requires N, 13·72%.)

A byproduct, m.p. 134-5°, identified as the *di*-(β-*phenylethyl*) *urea*, was also obtained. (Found: N, 10·46. $C_{17}H_{20}N_2O$ requires N, 10·44%.)

Acknowledgement

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Studies in Potential Amoebicides: Part XII—Synthesis of Some *p,p'*-Bis-(Dialkyl, Acyl or Arylamino)-*m,m'*-dimethyldiphenyl Methanes

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A number of *p,p'*-bis-(dialkyl, aryl or acylamino)-*m,m'*-dimethyldiphenyl methanes have been synthesized as potential amoebicides. None of these compounds, however, exhibits any marked activity against *Entamoeba histolytica*.

In pursuance of the suggestion made earlier[1] that the antiamoebic activity of conessine may be related among other factors to the electronic status and position of its two component tertiary nitrogen atoms, synthesis of a variety of ditertiary bases, in which the inter-nitrogen distance corresponds to that separating the two nitrogen atoms of conessine, has been reported[1-3]. Kaushiva[4] has shown that aliphatic ditertiary bases conforming to the above requirements have powerful antiamoebic properties *in vitro*. Since, however, the aliphatic amines are known to be bound strongly by plasma proteins, it was considered desirable to prepare suitably substituted aromatic tertiary diamines with appropriate intramolecular inter-nitrogen distances and test them for antiamoebic activity. The preparation of a number of substituted *p,p'*-diamino-*m,m'*-dimethyldiphenyl methanes is described in the present communication.

p,p'-Diamino-*m,m'*-dimethyldiphenyl methane was prepared according to Scanlan[5], and tosylated. The ditosyl compound was alkylated and this was followed by detosylation with acetic acid and hydrochloric acid mixture. The diacyldiamino compounds were prepared by direct acylation of the parent diamine. Mixed diamines, e.g. *p,p'*-di-(methylethylamino)-*m,m'*-dimethyldiphenyl methanes, were prepared by a two-step alkylation procedure involving the alkylation, or acylation, of *p,p'*-alkyl-(or acyl)-amino-*m,m'*-dimethyldiphenyl methanes.

For the preparation of the *p,p'*-diaryldiamino-*m,m'*-dimethyldiphenyl methanes, the parent diamine was condensed with appropriate aryl aldehydes and the Schiff bases so obtained were reduced catalytically in the presence of Raney nickel.

The various compounds now prepared, as also the corresponding intermediates, are recorded in Tables 1-4.

None of these compounds exhibited marked *in vitro* activity against *Entamoeba histolytica*. This inactivity may be due to the rather rigid spatial orientation of the molecule because of the presence of the interconnecting benzene rings.

Experimental procedure

p,p'-Diamino-m,m'-dimethyldiphenyl methane was prepared by the method of Scanlan[5].

p,p'-Di-(tosylamino)-m,m'-dimethyldiphenyl methane — *p,p'*-Diamino-*m,m'*-dimethyldiphenyl methane (22·6 g.) was suspended in dry pyridine (90 ml.) and *p*-toluene sulphonyl chloride (38·2 g.) was added gradually with occasional shaking. The reaction

mixture was left overnight and then poured into iced water. The separated solid (37 g.) was collected and crystallized from ethanol, m.p. 193°. (Found: N, 5·06. $C_{29}H_{30}N_2O_4S_2$ requires N, 5·24%.)

p,p'-Di-(methyltosylamino)-m,m'-dimethyldiphenyl methane — *p,p'*-Di-(tosylamino)-*m,m'*-dimethyldiphenyl methane (2·0 g.) was dissolved in alcohol (20 ml.), and aqueous potassium hydroxide (14 ml.; 20 per cent) and methyl iodide (1·2 g.) were added. The reaction mixture was heated on a water bath for 3 hr and worked up in the usual manner. The alkyl derivative (1·6 g.) was crystallized from ethanol, m.p. 160°. (Found: N, 4·88. $C_{31}H_{34}N_2O_4S_2$ requires N, 4·89%.)

The different alkyl tosyl derivatives prepared are listed in Table 1.

p,p'-Di-(methylamino)-m,m'-dimethyldiphenyl methane — *p,p'*-Di-(methyltosylamino)-*m,m'*-dimethyldiphenyl methane (1 g.), hydrochloric acid (3 ml.) and glacial acetic acid (10 ml.) were heated in a sealed tube at 100° for 12 hr. The reaction mixture was then poured into crushed ice and basified with sodium hydroxide. *p,p'*-Di-(methylamino)-*m,m'*-dimethyldiphenyl methane (0·6 g.) was crystallized from ethanol.

p,p'-Di-(acetylamino)-m,m'-dimethyldiphenyl methane — *p,p'*-Diamino-*m,m'*-dimethyldiphenyl methane (2·26 g.) and acetic anhydride (2·1 ml.) were refluxed together for 3 hr. After working up, the acetyl derivative (2 g.) was crystallized from ethanol, m.p. 190°. (Found: C, 75·70; H, 6·81; N, 8·19. $C_{21}H_{22}N_2O_2$ requires C, 75·45; H, 6·59; N, 8·38%.)

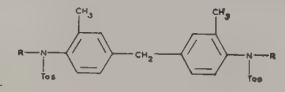
The different alkyl acyl derivatives so prepared are listed in Table 3.

p,p'-Di-(benzylideneamino)-m,m'-dimethyldiphenyl methane — A solution of freshly distilled benzaldehyde (3·36 g.) and *p,p'*-diamino-*m,m'*-dimethyldiphenyl methane (6·78 g.) in ethyl alcohol (100 ml.) was refluxed for 4 hr. The solvent was removed under reduced pressure and the residue crystallized from ethyl alcohol, m.p. 93°; yield 9·94 g. (Found: N, 7·09. $C_{29}H_{26}N_2$ requires N, 6·94%.)

The Schiff's bases obtained by condensation of the amines with different aldehydes are given in Table 2.

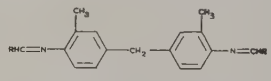
p,p'-Di-(benzylamino)-m,m'-dimethyldiphenyl methane — *p,p'*-Di-(benzylideneamino)-*m,m'*-dimethyldiphenyl methane (5 g.) was taken in ethanol (150 ml.) and hydrogenated at 60 p.s.i. in the presence of Raney nickel (1·5 g. per 5 g. of the substance) till the absorption of hydrogen was complete (4 hr). The catalyst was filtered off, the solvent removed under reduced pressure and the residue crystallized from ethyl alcohol, m.p. 107°; yield 4·62 g. (Found: N, 6·81. $C_{29}H_{30}N_2$ requires N, 6·79%.)

TABLE 1 — *p,p'*-DI-(TOSYLALKYLAMINO)-*m,m'*-DIMETHYLDIPHENYL METHANES



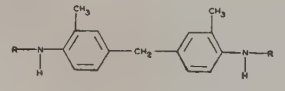
Sl No.	R	Yield %	M.P. °C.	Analysis: N, % Found	Req.
326	H	70	193	5·06	5·24
327	Methyl	80	160	4·88	4·89
328	Ethyl	72	145	4·47	4·74
329	*n*-Propyl	66	134	4·78	4·53
330	Isopropyl	63	141-2	4·62	4·53
331	*n*-Butyl	61	155	4·34	4·61
332	Allyl	55	132	4·93	4·56

TABLE 2 — *p,p'*-(DIARYLIDENEAMINO)-*m,m'*-DIMETHYLDIPHENYL METHANES



Sl No.	R	Yield %	M.P. °C.	Analysis: N, % Found	Req.
333	Phenyl	82·4	93	7·00	6·94
334	*p*-Hydroxyphenyl	76·4	67-68	6·56	6·43
335	*p*-Methoxyphenyl	77·5	137	6·31	6·06
336	3-Methoxy-4-hydroxyphenyl	73·8	186-7	6·05	5·70
337	3,4-Dimethoxyphenyl	77·3	145-6	5·83	5·40
338	3,4-Diethoxyphenyl	76·8	111-12	5·32	5·13

TABLE 3 — *p,p'*-DI-(MONO-SUBSTITUTED AMINO)-*m,m'*-DIMETHYLDIPHENYL METHANES



Sl No.	R	Yield %	M.P. °C.	Analysis: N, % Found	Req.
339	H	57·0	156-7	12·42	12·39
340	Methyl	65·0	86	10·81	11·02
341	Ethyl	52·0	85	9·80	9·93
342	*n*-Propyl	55·0	56	9·00	9·03
343	Isopropyl	58·0	60-61	9·02	9·16
344	*n*-Butyl	59·0	65	8·24	8·11
345	Acetyl	70·0	190	8·19	8·38
346	Benzoyl	50·0	210	6·29	6·45
347	Allyl	49·0	165	9·37	9·15
348	Benzyl	82·5	107-8	6·81	6·79
349	*p*-Hydroxybenzyl	86·3	Syrup	6·57	6·30
350	*p*-Methoxybenzyl	73·5	do	5·46	5·15
351	3-Methoxy-4-hydroxybenzyl	82·2	147-8	5·93	5·64
352	3,4-Dimethoxybenzyl	78·0	176-7	5·66	5·30
353	3,4-Diethoxybenzyl	81·1	123	4·85	4·81

The other Schiff's bases were reduced by the same procedure and the resulting products are listed in Table 3.

p,p'-Bis-(dimethylamino)-m,m'-dimethyldiphenyl methane — Formic acid (5·12 g.; 90 per cent) was added dropwise to *p,p'*-diamino-*m,m'*-dimethyldiphenyl methane (2·26 g.) under cooling and this was followed by the addition of formaldehyde solution (9 ml.; 37 per cent). The reaction mixture was then heated at 90-95° for 8 hr. After cooling, hydrochloric acid (10 ml.; 4*N*) was added and the solution evaporated to dryness under reduced pressure. The crystalline solid so obtained was dissolved in water (10 ml.) and sodium hydroxide (6 ml.; 18*N*) was added. The resultant solid (1·7 g.) was washed with water and crystallized from a mixture of benzene and alcohol, m.p. 122°. (Found: C, 80·74; H, 9·41; N, 10·12. $C_{19}H_{26}N_2$ requires C, 80·85; H, 9·22; N, 9·86%.)

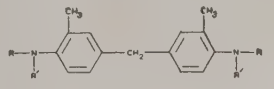
p,p'-Di-(N-methyl-N-ethylamino)-m,m'-dimethyldiphenyl methane — Sodium (0·1 g.) was dissolved in absolute alcohol (10 ml.) and *p,p'*-dimethylamino-*m,m'*-dimethyldiphenyl methane (1·1 g.) was added to it with stirring. Ethyl iodide (0·65 g.) was then added and the resulting solution heated under reflux for 6 hr. Alcohol was removed under reduced pressure and the reaction mixture was diluted with water and extracted with ether. The ethereal solution was dried (sodium sulphate) and the solvent removed. The residue (0·75 g.) was crystallized from ethanol, m.p. 105°. (Found: N, 8·90. $C_{21}H_{30}N_2$ requires N, 9·03%.)

p,p'-Bis-(diacetylamino)-m,m'-dimethyldiphenyl methane — *p,p'*-Diamino-*m,m'*-dimethyldiphenyl methane (2·26 g.) was refluxed with acetic anhydride (10 ml.) for 8 hr and the reaction mixture worked up in the usual manner. The solid so obtained (2 g.) was crystallized from ethanol, m.p. 120°. (Found: C, 70·26; H, 6·52; N, 7·48. $C_{23}H_{26}N_2O_4$ requires C, 70·00; H, 6·60; N, 7·10%.)

p,p'-Di-(N-benzyl-N-methylamino)-m,m'-dimethyldiphenyl methane — *p,p'*-Di-(benzylamino)-*m,m'*-dimethyldiphenyl methane (2·03 g.) was added slowly to formic acid (2·56 g.) with shaking and cooling. Formaldehyde solution (2·25 ml.; 37 per cent) was added to the resulting solution and the reaction mixture heated at 95-100° for 8 hr. The cooled solution was treated with hydrochloric acid (5 ml.; 4*N*) and evaporated to dryness under reduced pressure. The residue was dissolved in water (10 ml.), cooled and the solution made alkaline with sodium hydroxide solution (60 per cent) and extracted with benzene. The benzene extract was dried (potassium carbonate) and the solvent removed. A thick oily layer separated out which was characterized as picrate, m.p. 201-2°; yield 1·86 g. (Found: N, 12·81. $C_{31}H_{34}N_2$, $2C_6H_3N_3O_7$ requires N, 12·55%.)

p,p'-Di-(N-dichloroacetyl-N-3,4-diethoxybenzylamino)-m,m'-dimethyldiphenyl methane — Dichloroacetyl chloride (1·5 g.) in dry benzene was added slowly with stirring to an ice-cold mixture of *p,p'*-di-(3,4-diethoxybenzylamino)-*m,m'*-dimethyldiphenyl methane (1·18 g.) in dry benzene (20 ml.) and dry pyridine (0·5 ml.). The reaction mixture was allowed to come to room temperature gradually and stirring was continued for another 2 hr. Water (5 ml.) was added and the benzene layer washed with dilute sodium bicarbonate solution and then with water. The benzene solution was dried (anhydrous magnesium sulphate) and the solvent removed. The residue was crystallized from alcohol, m.p. 91-92°;

TABLE 4 — DI-(*p,p'*-DISUBSTITUTED AMINO)-*m,m'*-DIMETHYLDIPHENYL METHANES



Sl No.	R	R'	Yield %	M.P. °C.	Analysis: N, %	
					Found	Req.
354	Methyl	Methyl	62·0	122	10·12	9·86
355	do	Ethyl	50·0	105	8·90	9·03
356	do	*n*-Propyl	60·0	97	8·42	8·28
357	do	*n*-Butyl	65·0	89	7·83	7·65
358	*n*-Propyl	do	55·0	56	6·90	6·63
359	Acetyl	Acetyl	48·0	120	7·48	7·10
360	Benzoyl	Benzoyl	75·0	149-50	4·51	4·33
361	3,4-Dimethoxybenzyl	Dichloroacetyl	53·1	183-5	4·07	3·74
362	3,4-Diethoxybenzyl	do	51·6	91-92	3·75	3·48
363	Benzyl	Methyl	85·6	202-4 (picrate)	12·81	12·55

yleld 0·83 g. (Found: N, 3·75. $C_{41}H_{46}Cl_4N_2$ requires N, 3·47%.)

The various symmetrical and asymmetrical bis-(dialkyl or arylamino) derivatives are listed in Table 4.

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Studies on the Nutritional Aspects of Hydrogenated Tobacco Seed & Safflower Seed Oils

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Rats fed for 32 days on a synthetic diet containing 10 per cent fat have been found to exhibit the same weight gain and lipid absorption when the fat fed is groundnut oil, vanaspati (*Dalda*, a commercial brand of hydrogenated oil), hydrogenated tobacco seed oil or hydrogenated safflower seed oil. The lipid content in the faeces is also the same in all the groups of experimental animals indicating that the fats are absorbed to the same extent by the rats. These studies indicate that tobacco and safflower seed oils can be used in the manufacture of edible hydrogenated products.

PRODUCTS similar to commercial edible hydrogenated oils (vanaspati) in quality and consistency can be prepared by the hydrogenation of tobacco and safflower seed oils[1,2]. Though these hydrogenated products are similar in fatty acid composition to commercial vanaspati prepared from conventional oils, nevertheless it is desirable to carry out biological experiments to prove that the hydrogenated products from tobacco and safflower seed oils are non-toxic and safe when used for edible purposes. Studies have, therefore, been carried out to find out the effect of feeding rats with groundnut oil, a commercial brand of vanaspati and hydrogenated tobacco and safflower seed oils. The results are presented in this paper.

Experimental procedure

Female albino rats of different age groups, weighing between 60 and 104 g., were utilized for the experiment. Three to four rats were taken in each group and four such groups were employed. They were fed the following synthetic diet: starch, 66·0; fat-free casein, 18·0; salt mixture, 4·0; fat (control or experimental), 10·0; and yeast, 2·0 per cent.

The diet of the rats was supplemented by two drops of multivitamin (A, B, D, E and C; Parke-Davis & Co.) twice weekly. Groups I and II were fed on groundnut oil and vanaspati (a proprietory brand, *Dalda*, made by Hindustan Lever Ltd), and groups III and IV on hydrogenated safflower seed oil and tobacco seed oil respectively. The analytical data for the hydrogenated fats used in the experiments are given in Table 1.

The rats were weighed biweekly to observe their growth. The faeces were collected every day from the eighth day and the faeces from each group were pooled every week for the extraction of fats. The lipid matter was taken in thimbles and extracted

TABLE 1 — ANALYTICAL CHARACTERISTICS OF HYDROGENATED FATS

Fat	Slip point °C.	Iodine val. (Wij's 0·5 hr)	Fatty acid composition			
			Lino-leic %	*Cis*-oleic %	Iso-oleic %	Satu-rated %
Hydrogenated safflower seed oil	36·5	67·7	0·8	43·6	32·4	23·2
Hydrogenated tobacco seed oil	36·5	64·5	3·1	38·1	29·1	29·7
Dalda (control)	35·5	69·2	1·2	41·0	35·4	22·4

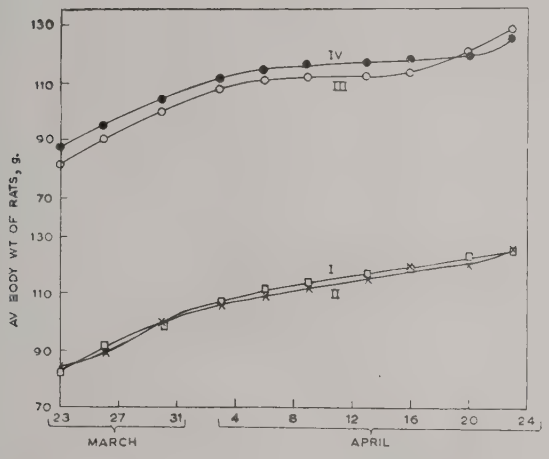

Fig. 1 — Growth curves showing the average body weights of different groups of rats fed on fats [I: groundnut oil; II: *Dalda*; III: hydrogenated safflower seed oil; and IV: hydrogenated tobacco seed oil]

with petroleum ether (40-60°C.) for 3-4 hr in a soxhlet apparatus. To each thimble containing faeces, about 10 ml. of 1:4 hydrochloric acid were added and allowed to stand for 1-2 hr. They were again extracted with petroleum ether for another 3-4 hr. The ether extract was freed of acid and the solvent distilled off to get the lipid.

The experimental animals were sacrificed after 32 days. The livers of the animals were weighed and pooled for each group. They were then cut into small pieces and 30 ml. of absolute alcohol, 15 ml. distilled water and 100 ml. petroleum ether were added in each case and transferred to a Waring blender and homogenized to a fine pulp. The homogenized mass was then transferred to a separating funnel and extracted with another 100 ml. portion of petroleum ether. The two extracts were mixed and the lipid matter isolated by removal of solvents.

Results and discussion

The results of growth studies presented in Fig. 1 show no appreciable difference between the control groups (groundnut oil and *Dalda*) and the experimental groups (hydrogenated safflower and tobacco seed oils). Studies on lipid excretion in different groups of rats (Table 2) show practically no difference in the amount of lipid in the faeces and, therefore, the fats are utilized to the same extent by the rats.

The lipid content of liver was slightly higher in the case of rats fed on groundnut oil (Table 3) but the livers of rats fed with *Dalda*, hydrogenated safflower

TABLE 2 — LIPID BALANCE IN RATS FED ON DIFFERENT FATS

Exptl period	Group of rats*	Food consumed g.	Amount of lipid in food consumed g.	Lipid in faeces g.	Lipid excreted/ day (on lipid intake) %
First week	I	209·0	20·90	0·9374	0·56
	II	141·0	14·10	0·9388	0·83
	III	205·0	20·50	0·9662	0·59
	IV	160·5	16·05	1·1420	0·71
Second week	I	213·5	21·35	1·0552	0·62
	II	144·4	14·40	0·6702	0·58
	III	209·0	20·90	0·8012	0·48
	IV	166·5	16·65	0·8086	0·60
Third week	I	178·5	17·85	0·5250	0·37
	II	133·0	13·30	0·6424	0·48
	III	172·0	17·20	0·6076	0·44
	IV	121·5	12·15	0·4846	0·50
Fourth week	I	181·5	18·15	0·5550	0·38
	II	128·0	12·80	0·6116	0·59
	III	223·5	22·35	0·6052	0·34
	IV	143·5	14·35	0·7232	0·63

*Group I: groundnut oil; group II: *Dalda*; group III: hydrogenated safflower seed oil; and group IV: hydrogenated tobacco seed oil.

TABLE 3 — LIPID CONTENT OF LIVERS OF RATS FED ON DIFFERENT FATS

Group of rats	No. of rats	Liver wt g.	Wt of liver lipids g.	Lipid in liver %
I	4	4·0, 4·8, 4·3, 3·7 (av. 4·2)	0·7086	4·22
II	3	6·0, 4·0, 5·5 (av. 5·2)	0·4720	3·04
III	4	3·0, 3·5, 3·5, 6·0 (av. 4·0)	0·4877	3·05
IV	3	3·0, 4·0, 4·0 (av. 3·7)	0·3429	3·12

Group I: groundnut oil; group II: vanaspati (*Dalda*); group III: hydrogenated safflower seed oil; and group IV: hydrogenated tobacco seed oil.

and tobacco seed oils contained almost the same amounts of lipid.

Contrary to popular belief, hydrogenated tobacco seed oil is, therefore, non-toxic and safe. Thus, safflower and tobacco seed oils could be hydrogenated and used just as other commercial edible hydrogenated products.

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Effect of Mounting Media on Quantitative Microscopy of Leaves

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When leaf fragments are mounted in different mounting media, their surface area becomes altered. Such changes in area affect quantitative microscopic data which are expressed as the concentration per unit area. The nature of contraction of fresh leaf pieces in different solvents and mounting media has been discussed. Dried leaf pieces when boiled in water do not expand fully; when cleared in chloral hydrate solution and mounted in Berlese mountant, the contraction of the leaf area is not as much as it is in water. Peeled epidermis is not significantly affected by different mounting media.

IN the quantitative microscopy of leaves, there are some constants which are expressed as the concentration of particular tissue per unit area of the lamina. But under the experimental conditions normally employed in the preparation and mounting of leaves, the surface area of the lamina may change. If the surface area of the lamina contracts, the component tissues become congested and the concentration is increased. So those constants which are expressed by the concentration per unit area like vein-islet number[1], veinlet termination number[2] and stomatal number[3] are affected due to such changes, whereas palisade ratio[4] and stomatal index[5], which give the relative proportions between two tissue systems, are not affected. The change of surface area may result from various causes like the drying of the leaves or by immersing them in solvents and mounting media of different osmotic values. There is no rigid schedule in the preparation of the materials for microscopic counting[6] and, therefore, the data may vary when the preparation of the slides is not identical.

Materials and methods

Healthy, normal and mature leaves from different members of the family *Solanaceae* were selected. The plants were grown in culture pots and were free from metabolic disorders. The collected leaves

were put into an isotonic solution (physiological saline) immediately after collection to maintain the turgidity of the cells. Small pieces were cut out from the central region of the lamina between midrib and margin, avoiding the thicker veins as far as possible. In each series containing five such pieces, only in one piece a thicker vein was allowed to pass parallel to one side. These cut pieces were spread over a slide with their upper epidermis on glass surface and then mounted in excess of saline and no pressure was applied on the materials in any step.

The outlines of the leaf pieces were drawn on a paper with the help of a camera lucida. These initial figures served as control. The materials were then washed in distilled water, adherent moisture was removed and put into solvents or mounting media or left to drying as the case may be. After the stipulated time the materials were again mounted with the liquid in which these were kept and the leaf pieces were drawn again as above over the first figure and the differences between the areas were noted. The averages of five such readings were recorded.

The change of area of the leaf piece was calculated by drawing the outline of the camera lucida drawings on a Whatman filter paper No. 1. The paper was cut along the outline and from the differences of the weights between the original and the changed areas of the drawings, the percentage of increase or decrease of area was calculated. It may be noted that by mounting the same piece of leaf several times and measuring it, the difference observed between maximum and minimum values was 3·2 per cent, which may be allowed as handling error. The data incorporated in the text represented the percentages of increase by (+) and decrease by (−). Due care was given to the evaporation of the solvents during the experiments.

Results

Effect of solvents and mounting media on fresh leaves — Leaves of different textures were used in the experiment. After initial reading, the leaves were kept in different solvents and mounting media overnight in plugged test tubes. After 20 hr of soaking, they were taken out and measured again. The results are recorded in Table 1.

In some cases it appeared that the absorption of the solvents or mounting media in which the cut leaves were immersed was not complete. So these were put into the same solvents or mounting media again and boiled in a water bath for 10 min. They

TABLE 1 — EFFECT OF SOAKING FRESH LEAVES IN DIFFERENT MEDIA

(*Soaking period,* **20** *hr; the values given in parentheses refer to leaves which were put into the same solvents and mounting media and boiled over a water bath for* 10 *min.*)

Plant	Physio. saline	Dist. water	Ethanol 95%	Chloral hydrate soln*	Glycerine 40%	Glycerine jelly*	Lacto-phenol*	Berlese mountant*
Cestrum diurnum	0·0	−0·4	−1·7	−0·4	−2·1	−4·5	−2·1	−1·9
	(+1·0)	(−0·4)	(−1·7)	(+0·7)	(−1·4)	(−6·7)	(−2·3)	(−1·9)
C. nocturnum	−0·8	−2·6	−3·2	−1·0	−3·8	−6·1	−4·1	−2·5
	(+0·3)	(−2·4)	(−4·2)	(−2·5)	(−7·3)	(−9·0)	(−4·6)	(−2·9)
Solanum nigrum	+4·8	+2·8	−6·1	−7·5	−17·5	−23·8	−17·2	−7·7
	(−3·1)	(−7·6)	(−10·6)	(−7·0)	(−15·6)	(−26·2)	(−12·5)	(−9·2)
S. verbascifolium	−4·1	−6·5	−5·4	−8·2	−11·0	−23·5	−12·0	−9·6
	(−7·1)	(−9·4)	(−13·1)	(−7·7)	(−17·8)	(−28·1)	(−10·7)	(−10·2)
S. torvum	−2·3	−2·6	−2·8	−4·5	−9·5	−19·6	−9·4	−5·8
	(−6·5)	(−7·2)	(−7·7)	(−7·8)	(−10·7)	(−18·5)	(−6·6)	(−9·6)
S. sisymbriifolium	−6·6	−6·1	−6·7	−7·0	−13·0	−14·0	−7·8	−8·0
	(−1·3)	(−6·4)	(−5·6)	(−3·9)	(−15·6)	(−16·9)	(−8·3)	(−10·6)
Physalis minima	−4·8	−5·5	−8·3	−5·8	−28·7	−40·2	−21·6	−10·0
	(−5·1)	(−10·0)	(−16·0)	(−8·0)	(−24·2)	(−40·5)	(−13·1)	(−12·1)
P. minima var. *indica*	−2·4	−1·5	−2·3	−1·7	−21·2	−33·3	−15·3	−7·2
	(−2·9)	(−6·6)	(−7·3)	(−6·9)	(−19·3)	(−35·2)	(−12·2)	(−12·7)
Withania somnifera	−1·1	+1·0	−4·6	−1·7	−8·9	−12·6	−4·5	−2·8
	(−4·9)	(−5·7)	(−5·6)	(−0·9)	(−10·3)	(−15·4)	(−5·3)	(−2·8)
Capsicum annuum	+1·3	−1·2	−4·4	−4·0	−8·4	−13·6	−6·3	−3·8
	(−3·8)	(−6·5)	(−7·0)	(−3·9)	(−9·6)	(−14·5)	(−5·0)	(−7·1)
Datura metel	−1·2	−1·2	−6·1	−4·5	−14·9	−17·7	−8·5	−9·2
	(−4·9)	(−4·8)	(−5·3)	(−3·0)	(−13·9)	(−24·2)	(−7·7)	(−8·5)
Nicotiana tabacum	+0·1	−3·2	−3·7	−5·4	−16·4	−36·2	−17·0	−10·8
	(−3·4)	(−7·1)	(−5·9)	(−4·7)	(−16·4)	(−36·2)	(−16·7)	(−12·5)
N. plumbaginifolia	−3·3	−3·8	−4·0	−3·4	−19·5	−36·7	−17·4	−9·3
	(+0·6)	(−7·0)	(−9·7)	(−3·6)	(−18·8)	(−39·6)	(−13·2)	(−14·3)

*Compositions of these media are from Wallis[8].
The composition of physiological saline (Locke) was: NaCl, 9 g.; KCl, 0·1 g.; $CaCl_2$, 0·2 g.; and distilled water, 1 litre.

were cooled and again mounted as before and measured. The results are given in Table 1 (values given in parentheses). After boiling, most of the leaves contracted, but a few became flaccid and tended to expand.

Contraction of area due to drying of leaves — When leaves are dried, the cells collapse. If the dried leaves are boiled in water, they never regain the original area. Experiments were, therefore, carried out with fresh leaves which were cut and measured in saline and then washed. After washing, the adherent moisture was removed by filter paper and dried in a forced circulation incubator kept at 40°C. After two days, when the leaf pieces were fully dried, they were taken out and boiled with distilled water in a water bath for 10 min. The materials were mounted with water and measured again. The percentage shrinkage in the leaves, calculated from the difference in the area of the leaf pieces before drying and after boiling, is given in Table 2.

Shrinkage of area when usual schedule for quantitative microscopy is followed — In quantitative microscopy, leaves or leaf fragments are usually cleared with chloral hydrate solution and then mounted in Berlese mountant. To investigate the extent of shrinkage in area in the leaf fragments due to this schedule, the following experiment was conducted. Fresh leaves were cut and measured as before and dried. The dried material was then boiled with chloral hydrate solution in a water bath for 10 min., cooled and mounted with Berlese mountant and the area measured again. The changes in the surface area noted are recorded in Table 2.

TABLE 2 — SHRINKAGE DUE TO DRYING AND TREATMENT WITH DIFFERENT MEDIA

Plant	Shrinkage due to drying %	Shrinkage due to treatment with media* %
Cestrum diurnum	−8·0	−1·3
C. nocturnum	−6·5	−3·6
Solanum nigrum	−30·6	−7·8
S. verbascifolium	−16·6	−6·9
S. torvum	−15·1	−4·4
S. sisymbriifolium	−19·3	−8·5
S. ferox	−18·2	−6·1
P. minima var. *indica*	−18·3	−9·4
Withania somnifera	−13·8	−4·7
Capsicum annuum	−14·3	−4·1
Datura metel	−19·9	−5·6
Nicotiana tabacum	−31·4	−9·8
N. plumbaginifolia	−37·3	−10·6

*Chloral hydrate and Berlese mountant schedule.

Change of area in epidermal layer — The epidermis of leaves, specially the upper one, is composed of stronger cells where there is no intercellular space and is resistant to deformity. But the rigidity of cell walls is not uniform and is dependent on the nature of the leaf, environment, maturity, etc. For ease of counting, often peeled epidermal layers are used in quantitative microscopy. To investigate the influence of some common mounting media on the peeled epidermis, the following experiments were conducted. Fresh leaves were cut and measured as before and then macerated with concentrated nitric acid till suitable for peeling. The upper epidermis was removed, freed from palisade cells, mounted with different mounting media and the area measured. The difference in area between the fresh leaf piece and its peeled epidermis in different mounting media is presented in Table 3.

To note the relative area of upper and lower epidermis, the epidermal layers were peeled from a leaf fragment of *Solanum torvum* and measured. It was found that the lower epidermis is 11·5 per cent bigger than the upper one. In the lower surface of the leaf the veins are raised, so that when the epidermis is removed and spread, the area appears to be bigger. For this reason only the upper epidermis was used. It is to be noted that peeled epidermis is very delicate and difficult to mount uniformly. It can be done by floating the epidermis in a shallow dish of water, scooping it out by a slide and removing the excess water. The mounting media are dropped on the slide and covered with a cover glass.

During this work it was observed that the leaf pieces of the same plant do not contract similarly

TABLE 3 — EFFECT OF MOUNTING MEDIA ON LEAF EPIDERMIS

Plant	Dist. water	Glycerine 40%	Glycerine jelly	Lactophenol	Berlese mountant
Cestrum diurnum	−4·8	−5·2	−4·4	−4·9	−4·7
C. nocturnum	−6·3	−6·5	−6·8	−7·3	−7·2
Solanum nigrum	−3·2	−4·4	−4·6	−3·2	−3·6
S. verbascifolium	−3·7	−4·8	−4·1	−3·0	−3·7
S. torvum	−7·5	−7·5	−8·5	−7·5	−10·0
Withania somnifera	−5·8	−6·8	−8·6	−7·7	−6·0
Capsicum annuum	−9·4	−9·3	−11·7	−10·6	−9·8
Nicotiana tabacum	−2·9	−4·8	−4·3	−2·4	−3·5

in the same immersing liquid. The veins do not contract appreciably and consequently the parenchyma cells around thicker veins contract less in surface area. Therefore, the sclerenchyma exerts some stabilizing effects on the contracting leaf pieces. The amount and nature of the sclerenchyma present in the leaf fragment are important factors in this respect. That is why wide differences were noticed between different readings in the same set of experiments. It was also noted that very old and yellow leaves contract less than the green but matured leaves. Immature leaves contract too much and so they were not used. In some cases the leaf pieces became crumpled in some solvents and mounting media could not be outlined properly.

Discussion

It appears from results given in Table 1 that physiological saline, distilled water, ethanol (95 per cent) and chloral hydrate solution cause little change in the surface area of leaf pieces. But all the mounting media tested [glycerine (40 per cent), glycerine jelly, lactophenol and Berlese mountant] have significant contracting effect on the leaf fragments. From the results given in Table 2 it appears that, on drying the leaves, some changes take place in the tissues, and by merely supplementing water the leaves do not regain their original size. But if the dried leaves are boiled in chloral hydrate solution and mounted in Berlese mountant (Table 2), the contracting effect on the leaf pieces can be reduced.

There are chances of errors due to handling while spreading and mounting the peeled epidermis (Table 3). The peeled epidermis obtained by acid treatment is a delicate object and is difficult to mount it properly. The epidermal layers are not affected to any significant extent by different mounting media, when sufficient care is taken in spreading and mounting the material.

Leaves of different species have been observed to behave differently when put into solvents or mounting media. A generalized rule in relation to the contracting power of different mounting media cannot, therefore, be formulated. As the use of different schedules for the preparation and mounting of leafy materials will result in differences in quantitative microscopic data, there is need for a standardized schedule.

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Rudanti: A Pharmacognostic Study—*Cressa cretica* Linn.

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Pharmacognostic study of *C. cretica*, known in indigenous medicine as *rudanti*, has been carried out because the exact identity of *rudanti* is still undecided. A similarity has been observed between the powder of this plant and that of *Evolvulus alsinoides* Linn., but the two drugs can be distinguished from each other by the following characteristics: only one type of unequally armed trichomes are seen on the stem of *E. alsinoides* whereas two types of trichomes, one ordinary and the other spindle-shaped, are observed on *C. cretica* stem. Calcium oxalate crystals are arranged along the veins in the leaves of *E. alsinoides* whereas the crystals are scattered at random throughout the leaves of *C. cretica*. The roots of *E. alsinoides* contain composite starch grains whereas individual starch grains are observed in the rhizome of *C. cretica*. *E. alsinoides* contains abundant spheroidal pollen grains whereas *C. cretica* powder does not usually contain pollen as the plant is collected before flowering.

CRESSA CRETICA Linn. (Sanskrit: *Rudravanti, rudranti, rudanti, sravanti*; Hindi: *Khardi*) is mentioned as an Ayurvedic drug, and is reported to be antibilious, antitubercular and expectorant[1-6]. The name *rudanti* has, during the past few years, appeared in the popular press as the Sanskrit equivalent for the plant *Capparis moonii* Wight. But the description given under *rudanti* in Ayurvedic literature[7], though scanty and general, would seem to apply rather to *C. cretica* than *Capparis moonii*, thus giving rise to much controversy in Ayurveda over the nomenclature of the drug. Further, *C. cretica* is described in all the present literature concerning indigenous drugs with one or the other vernacular name and all the properties mentioned above attributed to it, whereas the other claimant to the name *rudanti* is not listed as a drug at all[1-6]. It was, therefore, considered of interest to carry out pharmacognostic study of the two plants and explore the possibility of determining the identity of the drug *rudanti*, after subjecting the two drug plants to clinical trials.

C. cretica was collected locally from tank beds in December 1958, and dried and stored for about six months. Khory has stated that fresh drug should be procured for medicinal purposes[6]. The total ash content of *C. cretica* was found to be 12·97 per cent (moisture-free basis), of which 0·042 per cent was acid insoluble. The percentage extractives obtained with different solvents were: petroleum ether, 1·15; ether, 0·83; chloroform, 4·16; ethanol, 10·00; and water, 41·11.

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Description of the drug

Microscopic details

The drug consists of the whole plant. It is an erect herb, aerial part (Plate I, q) normally ranging from 10 to 20 cm. in height, but particularly healthy specimens in the drug may have a shoot system of about 30 cm. The underground part is a slender creeping rhizome. Up to ten branches may be found, covered with closely packed, tiny, deltoid leaves. The average internodal length is 3 or 4 mm. The whole drug shows an ashy white colour, with a slightly unpleasant odour and a salty taste.

Stem is ashy white, pubescent, smooth-surfaced, cylindrical, about 5-7 mm. in circumference; fracture short and equatorial.

Rhizome descending, straight or slightly tortuous at the tip, 7-8 mm. in circumference, light brown in colour, with fine parallel ridges running closely along to each other, scales present, at about 5 or 6 mm. interval, in the axil of which are buds. Adventitious roots are given out. There are thin transverse fissures in some. Rootlets and hairs present, fracture short, odour unpleasant, and taste salty (Plate I, r).

Leaves (Plate I, l) are usually 4-6 mm., or rarely 8 mm. long, densely pubescent, ashy white, deltoid, sessile, obtuse, entire, stipules O, slightly fleshy with

Plate I — Microscopic characters of stem, leaves, rhizome and powder of *C. cretica* [a and b: t.s. of stem × 106; c: epidermis of stem × 106; d: vessel from stem × 106; e: fibre tracheids from stem × 106; f: cortical cells of the stem containing chloroplasts × 200; g: tip of pericyclic fibre from stem × 200; h: ray cells from stem × 106; i: pit pairs × 396; j: cortical cells of stem × 106; k: cells of the pith of stem × 106; l: leaves × 4; m (left): starch grains from rhizome of stem × 200; m (middle): calcium oxalate crystals from phloem of stem × 200; m (right): calcium oxalate crystals from cortex of stem × 200; n, o and p: trichomes of stem epidermis × 106; q: aerial part of drug (2/5 natural size); r: rhizome found in the drug (2/5 natural size); s: calcium oxalate crystals and starch grains found in powdered material × 200; t: epidermal bits found in powder with crystals distributed × 44; u: t.s. of leaf through midrib and lamina × 106; and v: glandular hair from leaf epidermis × 106]

only the midrib faintly visible on the upper side and salty to taste.

Flowers are not seen in the drug but if they are present they are tiny, crowded on top of the stem, cupular and dull white in colour, with a faint pink flush on the inner surface of the petals.

Microscopic details

Stem — The transversely cut surface (Plate I, a and b) is about 1-2 mm. in diameter. Epidermis is a single layer of cubical cells, thin walled, outer walls convex. On surface view they are slightly longer than broad; cells measure R=10-**20**-40 μ, T=7-**15**-30 μ and L=30-**55**-85 μ. Stomata present, measuring 28×20 μ, glandular and covering two-armed trichomes, and oval scars indicating scars of fallen trichomes are seen (Plate I, c). Among the trichomes, two distinct sizes are made out. One has long unequal arms with their tips tapering finely. The longer arms measure 157-**645**-1000 μ, whereas the shorter ones measure 57-**186**-245 μ (Plate I, o). The other has shorter, stouter and slightly unequal arms and the whole trichome appears like a spindle on surface view. The longer measures 25-**51**-67 μ and shorter 15-**19**-28 μ. At the middle they are 20-35 μ wide (Plate I, n). Glandular trichomes have a short stalk and multicellular head, L=40-50 μ, and T=30-40 μ (Plate I, p).

The cortex has a radial depth of 85-**140**-175 μ, and is composed of two or three layers of chlorenchyma and the rest parenchyma. The chlorenchymatous layer immediately below the epidermis consists of radially elongated palisade-like cells measuring L=25-**35**-52 μ, and T=7-15 μ (Plate I, f). One or two layers that follow this and the rest of the cells of the cortex are polyhedral, thin-walled, transversely elongated, much crushed with small triangular intercellular spaces; they measure R=5-**12**-20 μ, T=25-**40**-55 μ and L=30-**60**-135 μ (Plate I, j). The last layer is in a row resembling the endodermis. Calcium oxalate rosette crystals of 10-14 μ diameter are found (Plate I, m, right). The pericycle consists of a broken ring of fibres, either in a linear row or in patches. They measure T=15-**25**-35 μ, R=15-20 μ and L=0·5-**1·5**-4 mm. The tips of some show branching (Plate I, g).

The stele is amphiphloic siphonostele. The external phloem is 25-**45**-100 μ radially and has sieve tubes, companion cells and parenchyma. Idioblasts containing rosette crystals of calcium oxalate of 6-8 μ in diameter (Plate I, m, middle) are present.

Xylem occupies a radial depth of 114-**170**-230 μ and is composed of vessels, tracheids, fibre tracheids and fibres. They are all arranged in neat radial rows, with the uniseriate or biseriate medullary rays occurring at every third or fourth radial row of xylem elements. Vessels are R=20-40 μ, L=70-**125**-256 μ and T=36-**55**-140 μ pitted with bordered, alternate pits, with a short ' tail ' at each end. The end-wall perforation is simple (Plate I, d). Fibre tracheids are 157-**315**-488 μ and T=5-**16**-20 μ, with irregularly sloping walls and a few slit-like pits down its middle (Plate I, e). Fibres measure L=355-**960**-1320 μ and T=10-14 μ. Ray cells are pitted, 50-100 μ long and 12 μ broad (Plate I, h). Pith cells are L=40-**85**-130 μ and T=19-**40**-70 μ (Plate I, k), with rosette crystals of calcium oxalate of diameter 9-**14**-20 μ.

Rhizome — The transverse section (Plate II, a and b) shows an irregular outline with a diameter of 1·5-2·0 mm. and has the following regions: the single layer of epidermis is unruptured, inner tangential wall lignified, cells R=15-25 μ, T=7-**13**-24 μ and L=75-**120**-165 μ; there are two layers below the epidermis of radial depth 40-50 μ, having thick lignified inner tangential walls with simple pits that are seen in both transverse and macerated materials. These cells measure L=20-**50**-80 μ and T=15-**24**-36 μ (Plate II, e). This is followed by a cortex, about 140-345 μ long radially, consisting of polyhedral, crushed, thin-walled parenchymatous cells with little intercellular spaces (Plate II, g). They measure L=39-**110**-210 μ and R=23-**32**-60 μ, and contain plenty of oval and circular starch grains. The oval grains measure L=12-16 μ and T=8-12 μ, and the circular are 10-12 μ in diameter (Plate I, m, left). Distributed here and there in the cortex are resin cells and stone cells of irregular shape, pitted walls, R=25-50 μ, T=60-100 μ and L=100-350 μ (Plate II, i). The pericycle consists of a broken ring of much smaller groups of fibres and much farther apart than those in the stem. Each group has three or four fibres. They are 14-18 μ broad and have a flattened, rather than tapering, tips. They are nearly 2·5 mm. long, but short ones of 300-500 μ length are also found. External phloem is radially 70-75 μ deep, and has sieve tubes, companion cells and parenchyma. Idioblasts containing calcium oxalate crystals of diameter 6-10 μ are found (Plate II, j).

Xylem occupies a radial depth of 240-330 μ, and is composed of vessels, tracheids, fibre tracheids and fibres. All xylem elements have bordered pits which are mere slits in the fibre tracheids and absent from the fibres. Pit pairs are seen both in the transverse (Plate II, k) and in the macerated material. The vessels measure L=100-**160**-300 μ and T=40-**48**-70 μ, with a short, sharp ' tail ' (Plate II, f). Fibre tracheids are abundant, measuring L=140-**460**-1000 μ and T=8-**16**-20 μ. Ray cells are pitted,

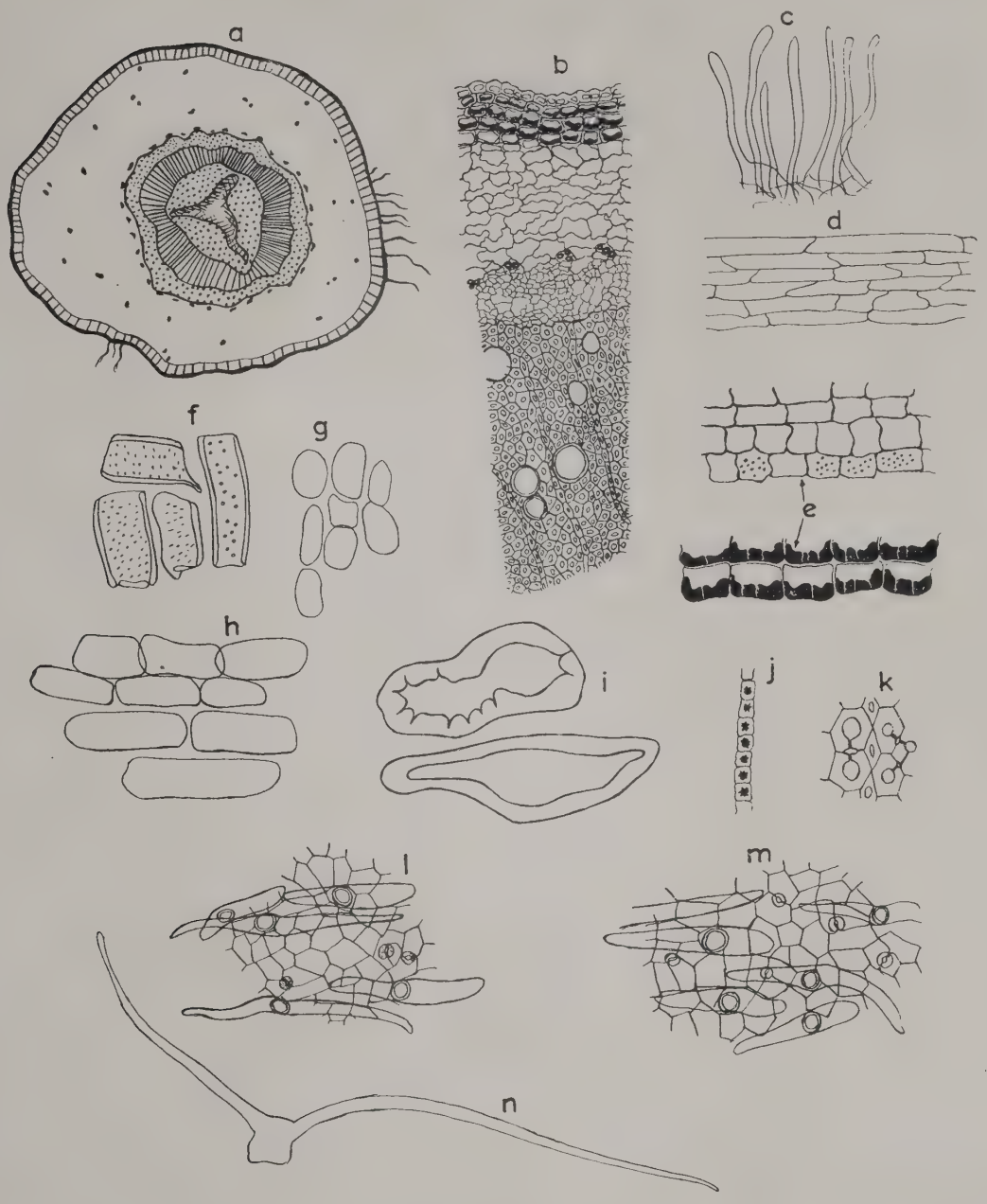

Plate II — Microscopic characters of rhizome and leaf of *C. cretica* [a and b: t.s. through rhizome × 30; c: hair-like roots from rhizome × 132; d: epidermis of rhizome × 132; e: cells below epidermis (× 265) with pitted walls (× 132) of rhizome × 132; i: stone cells from the cortex of rhizome × 250; j: idioblasts from the phloem of rhizome × 250; k: pit pairs in t.s. of rhizome × 495; l: upper epidermis of leaf × 132; m: lower epidermis of leaf × 132; and n: trichome of leaf × 132]

L=40-**63**-75 μ and T=9-12 μ. Pith cells are L=47-**96**-142 μ and T=23-**31**-52 μ (Plate II, h).

Leaf — There is a thin cuticle on both the surfaces, and the stomata of the lower are slightly larger than those of the upper. Epidermal cells slightly longer than broad, polyhedral, walls not wavy, tannin present, glandular and ordinary trichomes present (Plate II, l and m), the former with an oblong multicellular head filled with brownish contents. Stalk cells are as broad as the head, but distinguished from it by its lack of contents. The shorter arm of the ordinary trichome has a length range of 50-300 μ, while that of the longer is 200-400 μ (Plate II, n).

In the transverse section the midrib region is not noticeably broader than the laminar, the breadth being 250-340 μ from upper to lower epidermis, and the leaf margin is rounded (Plate I, u). Bundles in the lamina are few and those in the midrib show the usual elements and a few fibres surrounding it. Leaf is isobilateral, palisades appearing both below the upper and above the lower epidermises at both the midrib and the laminar regions. A certain amount of spongy parenchyma appears in the middle of the lamina and these are not clearly isodiametric, as cells of the spongy parenchyma usually are, but slightly elongated instead.

Epidermal cells in the transverse section appear cubical, outer walls convex, cuticle thin, ordinary trichomes on par with the neighbouring cells but the basal cell of the glandular trichome well sunk. Calcium oxalate rosette crystals are distributed throughout the mesophyll tissue (Plate I, t). They are 8-12 μ in diameter. Palisade cells range in length from 20 to 40 μ and breadth 8 to 12 μ. The stomatal cells are on par with the cells of the epidermis.

Powder — The powder of this plant resembles the powder of *Evolvulus alsinoides*[8] in certain important respects: they are both identical in colour, with trichomes rolling up into fluffy tiny balls, and react similarly with dilute sulphuric, hydrochloric and nitric acids and other reagents. Both have the same types of glandular and armed ordinary trichomes, and both leaves contain calcium oxalate rosette crystals, and starch grains occur in both. But close observation permits distinction and diagnosis, on the basis of Table 1.

Though the last three characteristics of the powders (Table 1) seem to present more conclusive evidences as to the identity of the drugs, they are not dependable for the reason that in a fine powder one rarely finds bits large enough to show the secretory canals or the bundle ends clearly. Starch grains, trichomes and calcium oxalate crystals, however, will be present even in the finest powders, and are, therefore, more reliable diagnostic features.

TABLE 1 — DISTINGUISHING CHARACTERS OF THE POWDERS OF *E. ALSINOIDES* AND *C. CRETICA*

	E. alsinoides	*C. cretica*
1.	On the stem are seen unequally armed trichomes of only one type; short arm 70-**200**-220 μ and long arm 500-**680**-800 μ	Two types of trichomes are seen on the stem; the long arm in one measures 157-**645**-1000 μ and the short arm 57-**186**-245 μ; other trichome is spindle-shaped
2.	Calcium oxalate crystals in leaves arranged along the veins and not scattered all over; they measure up to 20 μ in diam.	Calcium oxalate crystals scattered at random throughout the lamina and do not exceed 15 μ in diam. (Pl. I, s and t)
3.	Root consists of composite starch grains of diam. 8-10 μ	Individual starch grains of diam. 8-12 μ present in rhizome (Pl. I, s)
4.	Abundant spherical pollen grains present	Pollen grains not usually found since the plant is collected before flowering
5.	Abundant secretory canals	No secretory canals in leaves
6.	Epidermal cell walls of leaf sinuous	Epidermal cell walls of leaf not sinuous
7.	Characteristic bundle ends present	No characteristic bundle ends present

Acknowledgement

We are grateful to Dr Jivraj N. Mehta, Chairman, Research Subcommittee, Seth U.P. Ayurvedic Research Unit, and Dr J. D. Pathak, Dean of the Medical College, Baroda, for their keen interest in this work.

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Short Communications

Digitalis-like Activity of Glycosides from *Vallaris solanacea*

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Evidence of positive inotropic activity in the glycoside mixture obtained from leaves of *Vallaris solanacea* has been obtained on hypodynamic guinea-pig heart. The ECG changes produced in cats are typical of a cardinolide. The glycosides produce emesis in pigeons and show general musculotropic activity.

IN course of our investigations on cardiac glycosides from indigenous plants, *Vallaris solanacea* O. Katze belonging to the natural order *Apocynaceae* was taken up for pharmacological investigation. Jamwal *et al.*[1], in a recent study on the pharmacological activity of glycosides derived from the leaves of this plant, stated that " the mixture of glycosides possesses a stimulant action on all involuntary muscles especially on the uterine muscle and exhibits no digitalis-like activity". However, as a result of our preliminary study we find that the mixture of glycosides derived from the leaves of *V. solanacea* possesses a definite and a powerful digitalis-like activity, besides its general musculotropic activity on smooth muscle. The latter property is a common factor to all cardiac glycosides. The results of this study are reported in this communication. Detailed report will be published in due course.

The leaves were extracted with alcohol, the extract concentrated under vacuum, diluted with water and extracted with petroleum ether followed by chloroform. The mixture of glycosides obtained from the chloroform extract was used in the present study.

A stock solution of 10 mg./ml. of crude glycoside mixture was prepared in 25 per cent alcohol. Dilutions of the required strength were prepared with normal saline just before the experiment.

The evidence for cardiotonic activity was obtained by the following methods:

Hypodynamic guinea-pig heart — Isolated heart was perfused with Kreb-Ringer bicarbonate solution

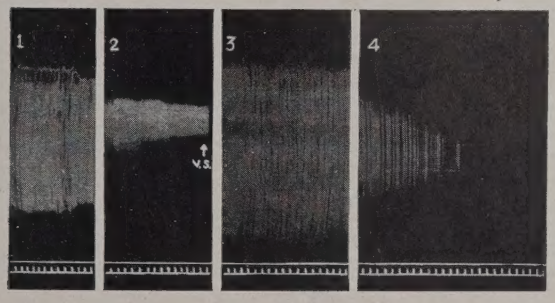

Fig. 1 — Guinea-pig heart, Langendorf preparation [(1) Normal amplitude of contraction; (2) hypodynamic condition after 4 min. of perfusion with half calcium fluid; (3) peak inotropic effect showing slow and strong heart after 9 min. of perfusion; and (4) irregular heart ending in standstill after 12 min. of perfusion]

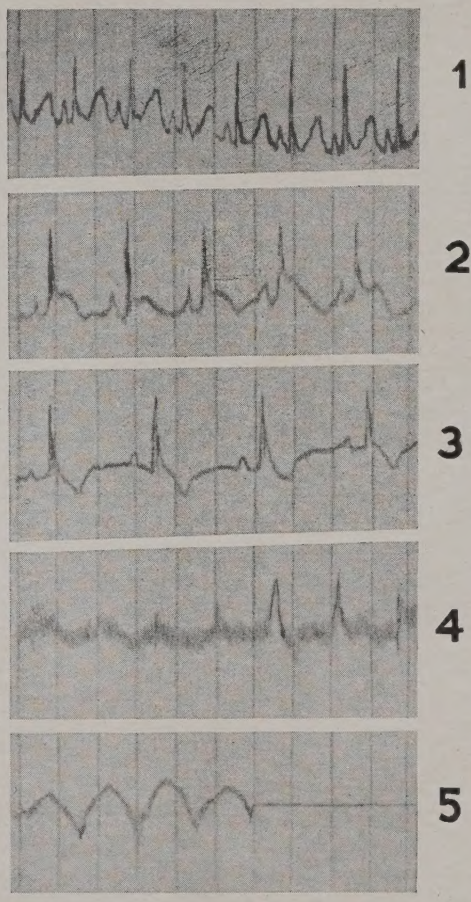

Fig. 2 — Experiment 98/60: ECG record of a cat anaesthetized with pentobarbital and perfused with glycoside mixture obtained from *V. solanacea* (250 µg./ml./min.); speed 30 mm./sec.; calibration 1 mV. = 1 cm. [(1) Control record: frequency 220/min.; T upright; regular sinus rhythm; (2) 13 ml.: frequency 160/min.; regular sinus rhythm with sagging of ST segment; (3) 20 ml.: frequency 120/min.; marked P-R prolongation; inversion of T-wave; sinus rhythm; (4) 31 ml.: auricular ventricular dissociation; ventricular tachycardia; and (5) 42 ml.: ventricular fibrillation and cardiac standstill]

containing 0·1 per cent glucose. The method of Vick and Kahn[2] was employed for rendering the heart hypodynamic. A solution containing half the quantity of calcium chloride but made isotonic by equivalent amount of sodium chloride was used. The heart became hypodynamic within 5 min. of perfusion with this fluid. At this stage the perfusion was switched over to the fluid (low calcium) containing the mixture of glycosides (100 μg./ml.). The heart began to improve its force of contraction within 2-3 min. and showed a marked inotropic activity within 5-6 min. of perfusion. The maximum inotropic effect persisted till 10 min., when the heart developed irregularities and ultimately stopped in systole in 12-14 min. Similar results were obtained in 4 experiments (Fig. 1).

Electrocardiographic changes — The effects on ECG records were determined in anaesthetized cats. The mixture of glycosides in a concentration of 250 μg./ml. was slowly perfused (1 ml./min.) intravenously. ECG was recorded on lead II in all cases. Effects typical of a cardiac glycoside such as bradycardia, prolongation of P-R interval, inversion of T-wave, ventricular tachycardia, ventricular fibrillation ending in cardiac standstill were noted (Fig. 2).

From these studies it was also found that slowing was evident when 20-25 per cent of the lethal dose had been given. The dosage causing cardiac irregularities was about 50-60 per cent of lethal dose. Percentage of bradycardia obtained in these experiments was about 35-45 per cent. Blood pressure record showed a slow but marked rise.

Emesis — The emetic effect of glycoside mixture was studied in pigeons. The drug was injected intravenously and the birds kept individually in cages for observation. The glycoside mixture produced 100 per cent emesis instantaneously at the dosage level of 0·25-0·5 mg. per bird. All the birds died within 5 min. of injection when 1 mg. per bird was injected intravenously.

Effect on smooth muscle — Musculotropic activity of crude glycosides was studied *in vitro* on the guinea-pig ileum and uterus in a 50 ml. Dales bath. Concentrations of 1 μg./ml. of the mixture caused a contraction in either case. The spasmogenic effect of acetyl choline on ileum was decreased following pretreatment with the glycosides, but no such decrease in effect of syntocinon on the uterus was noticed.

The biological activity of crude glycoside mixture was determined by the conventional methods. The Hatcher dose was found to range between 2·6 and 3·7 mg./kg. when determined in four cats of either sex weighing between 2·5 and 3·5 kg. anaesthetized with pentobarbitone. Concentrations of the perfusate were so adjusted that the time taken to bring about the cardiac standstill was within 30-45 min. *British Pharmacopoeia* (1958) method was employed for assay in guinea-pigs. Lethal dose determined in guinea-pigs ranged between 4·8 and 6·3 mg./kg. in six male guinea-pigs weighing 350-550 g. Cardiac arrest in guinea-pigs was determined by thrusting a needle through the chest wall over the heart. Rate of infusion was such as to cause death in 15-20 min.

We wish to thank Dr B. Mukerji for his kind interest and Dr Nitya Anand for kindly supplying the material used in this study.

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